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(54) Title: MYCOBACTERIUM TUBERCULOSIS GENES ENCODING PROTEIN ANTIGENS

(57) Abstract

Mycobacterium tuberculosis genes encoding five immunologically relevant proteins have been isolated by systematically screening a lambda gt11 recombinant DNA expression library with a collection of murine monoclonal antibodies directed against protein antigens of this pathogen. One of the *M. tuberculosis* antigens, a 65kD protein, has been shown to have determinants common to *M. tuberculosis* and *M. leprae*. In addition, genes encoding proteins of other mycobacteria (*M. africanum*, *M. smegmatis*, *M. bovis* BCG and *M. avium*) have been isolated. Isolation and characterization of genes encoding major protein antigens of *M. tuberculosis* make it possible to develop reagents useful in the diagnosis, prevention and treatment of tuberculosis. They can be used, for example, in the development of skin tests, serodiagnostic tests and vaccines specific for tuberculosis.

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MYCOBACTERIUM TUBERCULOSIS GENES AND
ENCODING PROTEIN ANTIGENS

Description

Background

05 Tuberculosis was the major cause of infectious
mortality in Europe and the United States in the
19th and early 20th centuries. Dubos, R. and J.
Dubos, The White Plague: Tuberculosis, Man and
10 Society, Little Brown & Co., Boston, MA, (1952).
Today, it remains a significant global health
problem.

For example, in the United States there are
over 20,000 new cases of tuberculosis diagnosed
annually. In addition, the steadily declining
15 incidence of tuberculosis evident in preceding years
appears to have changed course, reaching a plateau
in 1985 and showing an increase in the first half of
1986. Centers for Disease Control, Morbidity/Mor-
20 tality, Weekly Report, 34:774 (1986); and Centers
for Disease Control, Morbidity/Mortality, Weekly
Report, 35:774 (1986).

Worldwide, tuberculosis remains widespread and
constitutes a health problem of major proportions,
particularly in developing countries. The World
25 Health Organization estimates that there are ten
million new cases of active tuberculosis per year
and an annual mortality of approximately three

-2-

million. Joint International Union Against Tuberculosis and World Health Organization Study Group, Tubercle, 63:157-169 (1982).

05 Tuberculosis is caused by Mycobacterium (M.)
tuberculosis or Mycobacterium (M.) bovis, which are
the 'tubercle bacilli' of the family Mycobacteri-
aceae. M. bovis is a species which causes tubercu-
10 losis in cattle and is transmissible to humans and
other animals, in whom it causes tuberculosis. At
present, nearly all tuberculosis is caused by
respiratory infection with M. tuberculosis. Infec-
tion may be asymptomatic in some, but in other
15 individuals, it produces pulmonary lesions which
lead to severe debilitation or death. Resistance to
tuberculosis is provided by cell-mediated immune
mechanisms.

Mycobacteria are aerobic, acid-fast, non-spore-
forming, non-motile bacilli with high lipid contents
and slow generation times. M. leprae is the eti-
20 ologic agent of leprosy and, among the other myco-
bacteria, the only major pathogen. Bloom, B.R. and
T. Godal, Review of Infectious Diseases, 5:765-780
(1983). However, other mycobacterial species are
capable of causing disease. Wallace, R.J. et.al.,
25 Review of Infectious Diseases, 5:657-679 (1984).
M. avium, for example, causes tuberculosis in fowl
and in other birds. Members of the M.
Avium-intracellularae complex have become important
pathogens among individuals with acquired immuno-
30 deficiency syndrome (AIDS). Certain groups of

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individuals with AIDS have a markedly increased incidence of tuberculosis as well. Pitchenik, A.E. et. al., Annals of Internal Medicine, 101:641-645 (1984).

05 Diagnostic and immunoprophylactic measures for mycobacterial diseases have changed little in the past half century. Tuberculin, developed by Koch as a cure for tuberculosis in the late 1800s, is an M. tuberculosis filtrate of complex and poorly-defined
10 composition. It is used as a skin test antigen to detect prior exposure to the bacillus. Enrichment of the protein fraction of this material in the 1930's produced the purified protein derivative (PPD) which is still used to diagnose exposure to
15 tuberculosis. Seibert, F.M. et.al., American Review of Tuberculosis, 30(Suppl.):705-778 (1934). Its usefulness is limited, however, by its lack of specificity and its inability to distinguish active
20 disease from prior sensitization by contact with M. tuberculosis or cross-sensitization to other mycobacteria. Young, R.A. and R.W. Davis, Proceedings of the National Academy of Sciences, USA, 80:194-1198 (1983).

25 Bacille Calmette Guerin (BCG), an avirulent strain of M. bovis, has been used widely as a live vaccine against tuberculosis for over 50 years. Calmette, A., C. et.al., Bulletin of the Academy of

-4-

05 Medicine Paris, 91:787-796 (1924). During that
time, numerous studies have shown that BCG has
protective efficacy against tuberculosis. These
studies are reviewed by F. Luelmo in American Review
10 of Respiratory Diseases, 125(pt. 2):70-72 (1982).
However, more recently, a major trial of BCG in
India indicated that such a vaccine was not protec-
tive against tuberculosis in this setting. World
15 Health Organization WHO Technical Report Series, 651
(1980). Presently available approaches to diagnos-
ing, preventing and treating tuberculosis are
limited in their effectiveness and must be improved
if a solution is to be found for the important
public health problem tuberculosis represents
worldwide.

Summary of the Invention

The present invention is based on the isolation
of genes encoding immunogenic protein antigens of
the tubercle bacillus Mycobacterium tuberculosis (M.
20 tuberculosis). Genes encoding such protein antigens
have been isolated from a recombinant DNA expression
library of M. tuberculosis DNA. Genes encoding
proteins of four additional mycobacteria have also
been isolated and restriction maps produced.

25 In particular, genes encoding five immunodomi-
nant protein antigens of the tuberculosis bacillus
(i.e., those M. tuberculosis proteins of molecular
weight 12,000 daltons (12kD), 14kD, 19kD, 65kD and
71kD have been isolated by probing a lambda gt11
30 expression library of M. tuberculosis DNA with

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monoclonal antibodies directed against M. tuberculosis-specific antigens.

Recombinant DNA clones producing the specific antigenic determinants recognized by the monoclonal antigens were also isolated in this manner. DNA from such recombinant lambda gt11 clones was mapped with restriction endonucleases; the restriction maps for genes encoding the five immunodominant protein antigens (i.e., genes encoding the 12kD, 14kD, 19kD, 65kD and 71kD proteins) were deduced. The nucleotide sequence of three of the genes have been determined and, in each case, the amino acid sequence of the encoded protein has been deduced.

Brief Description of the Drawings

Figure 1 shows restriction maps of M. tuberculosis DNA. Recombinant DNA clones isolated with monoclonal antibodies directed against the 12kD, 14kD, 19kD, 65kD and 71kD protein antigens were mapped with restriction endonucleases. The insert DNA endpoints are designated left (L) or right (R) in relation to lac Z transcripts which traverse the insert from right to left. Restriction sites are represented as follows: A, Sal I; B, BamHI; E, EcoRI; G, BglIII; K, KpnI; P, PvuI; S, SacI; X, XhoI.

Figure 2 shows arrays of antigens from M. tuberculosis recombinant DNA clones probed with rabbit hyperimmune serum. The code of the recombinant DNA clones shown on the numbers filter is: 1, Y3275; 2, Y3274; 3, Y3279; 4, Y3277; 5, Y3247; 6, Y3272; 7, Y3150; 8, Y3254; 9, Y3147; 10, Y3163; 11,

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Y3179; 12, Y3191; 13, Y3252; 14, Y3178; 15, Y3180; 16, Y3143; 17, lambda gt11. Clones 1, 5, 6, 7, 9 and 16 are M. tuberculosis recombinants described in the following section. Clones 10, 11, 14 and 15 are M. leprae recombinants expressing epitopes of the 18kD, 28kD, 36kD and 65kD antigens, respectively. Clones 2, 3, 4, 8, 12, 13 are uncharacterized recombinants from the lambda gt11 M. tuberculosis and M. leprae libraries. Clone 17 is a non-recombinant lambda gt11 control.

Figure 3 shows arrays of recombinant mycobacterial antigens probed with monoclonal antibodies to assess the extent of cross-reactivity between recombinant protein antigen of M. tuberculosis and of M. leprae. The array of clones is identical to that shown in Figure 2. Antibody probes and the antigen sizes recognized are: 1, IT-11 (71kD); 2, IT-31 (65kD); 3, IT-16 (19kD); 4, IT-1 (14kD); 5, IT-3 (12kD).

Figure 4 shows restriction maps of DNA encoding four proteins (71kD, 65kD, 19kD and 14kD) of M. tuberculosis and four proteins (71kD, 65kD, 19kD and 14kD) of M. bovis BCG. Restriction sites are represented as follows: A, AatII; B, BamHI; C, BclI; D, DraIII; E=EcoRI; G, BglII; H, HinfI; K, KpnI; P, PstI; S, SalI; V, PvuI and X, XhoI.

Figure 5 is a comparison of restriction maps of the gene encoding the 65kD protein of 6 mycobacteria (M. leprae, M. tuberculosis, M. africanum, M. bovis BCG, M. smegmatis, M. avium). Restriction sites are

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as follows: B, BamHI; K, KpnI; N, SacI; P, PvuI; S, SalI; X, XhoI.

Figure 6 is the nucleotide sequence of the region containing the M. tuberculosis 19kD gene.
05 The deduced amino acid sequence of the encoded protein is also represented (protein start position, nucleotide 1110; protein stop position, nucleotide 1586).

Figure 7 is the nucleotide sequence of the region containing the M. tuberculosis 71kD gene and the deduced amino acid sequence of the encoded protein.
10

Figure 8 is the nucleotide sequence of the region containing the M. tuberculosis 65kD gene.
15 The deduced amino acid sequences of the two long open reading frames are presented in one letter code over (540) or under (517) the appropriate triplets.

Detailed Description of the Invention

The invention described herein is based on the isolation of genes encoding immunogenic protein
20 antigens of the bacillus M. tuberculosis, which is the major etiologic agent of tuberculosis. In particular, it is based on the isolation, using monoclonal antibodies directed against M.
25 tuberculosis-specific antigens, of genes encoding five immunogenic protein antigens of the tuberculosis bacillus; these five antigens are immunodominant. Immunogenic antigens are those which elicit a response from the immune system.
30 Immunodominant protein antigens are immunogenic

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antigens against which the immune system directs a significant portion of its response. Genes encoding M. tuberculosis antigens of molecular weight 12,000 daltons (12kD), 14kD, 19kD, 65kD and 71kD were
05 isolated in this manner.

Isolation and characterization of major protein antigens of M. tuberculosis, as described herein, make it possible to develop more effective tools for the prevention, diagnosis, and treatment of tubercu-
10 losis. Identification and isolation of genes encoding five immunodominant M. tuberculosis protein antigens, as well as of the five protein antigens, are described below; uses of the genes and encoded products are also described.

15 M. bovis BCG DNA clones were also isolated for the genes encoding the 71kD, 65kD, 19kD and 14kD proteins. In order to compare M. bovis BCG and M. tuberculosis genes encoding proteins of similar molecular weight, restriction endonuclease maps were
20 determined for DNA segments containing each of the genes. Restriction maps for each of these genes is represented in Figure 4.

In addition, DNA clones were isolated for the genes encoding the 65kD protein from M. africanum,
25 M. smegmatis and M. avium. Restriction endonuclease maps were determined for DNA segments containing each of these genes. The restriction maps for these genes, as well as for the genes encoding the 65kD protein of M. tuberculosis, M. bovis BCG and M. leprae,
30 are represented in Figure 5.

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I. Construction of a recombinant expression library of M. tuberculosis DNA

A recombinant DNA expression library of M. tuberculosis DNA was constructed using lambda gt11. The library was constructed with M. tuberculosis genomic DNA fragments in such a way that all protein-coding sequences would be represented and expressed. Young, R.A., B.R. Bloom, C.M. Grosskinsky, J. Ivanyi, D. Thomas and R.W. Davis, Proceedings of the National Academy of Sciences, USA, 82:2583-2587 (1985).

Lambda gt11 is a bacteriophage vector which is capable of driving the expression of foreign insert DNA with E. coli transcription and translation signals. Lambda gt11 expresses the insert DNA as a fusion protein connected to the E. coli Beta-galactosidase polypeptide. This approach ensures that the foreign DNA sequence will be efficiently transcribed and translated in E. coli. This approach is also useful in addressing the problem of the highly unstable nature of most foreign proteins; fusion proteins are often more resistant to proteolytic degradation than is the foreign polypeptide alone. Lambda gt11 and the E. coli strain used (Y1090) have been described previously. Young, R.A. et al., Proceedings of the National Academy of Sciences, USA, 80:1194-1198 (1983); Young, R.A. and R.W. Davis, Science, 222:778-782 (1983). The teachings of these publications are incorporated herein by reference. The library constructed in this manner has a titer of 1×10^{10} pfu/ml. and

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contains approximately 40% recombinants with an average insert size of 4kB.

II. Screening of the lambda gt11 M. tuberculosis library with antibody probes

05 Murine monoclonal antibodies to protein antigens of M. tuberculosis were used individually to probe the M. tuberculosis recombinant DNA library. This work is described below and with specific reference to the 65kD antigen in the Exemplification. The antibodies used as probes and the sizes of the antigens to which they bind are shown below.

		<u>M. tuberculosis</u>	
		<u>Antibody</u>	<u>Antigen</u>
15		IT-3	12kD
		IT-20	14kD
		IT-19	19kD
		IT-27	19kD
		IT-17	23kD
20		IT-29	23kD
		IT-15	38kD
		IT-21	38kD
		IT-23	38kD
		IT-13	65kD
25		IT-31	65kD
		IT-33	65kD
		IT-11	71kD

Engers, H.D. et al., Infectious Immunology,
51:718-720 (1986).

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All monoclonal antibodies were used at approximately 1:200 to 1:300 dilution in 50mM Tris-HCl pH8/150 mM NaCl/.05% Tween 20.

05 Screening of the lambda gt11 recombinant DNA library was performed as described by Young et al. in Proceedings of the National Academy of Sciences, USA, 82:2583-2587 (1985), the teachings of which are incorporated herein by reference. One modification was made in the method described by Young and
10 co-workers: 1% bovine serum albumin was used in place of 20% fetal calf serum to decrease background.

Briefly, cloned lambda gt11 recombinants were arrayed on lawns of E. coli Y1090. The phage were
15 grown, antigen expression was induced and the antigens were blotted and probed with serum. Detection of signal-producing plaques was performed with a biotinylated secondary antibody system (Vectastain, Vector Laboratories, Burlingame, CA) or
20 with an alkaline phosphatase conjugated secondary antibody system (Protoplot, Promega Biotec, Madison, WI), both used according to manufacturer's instructions. Signal-producing clones were isolated using antibodies directed against protein antigens of
25 molecular weight 12kD, 14kD, 19kD and 65kD and 71kD. In each case, similar numbers of clones were isolated in screens of approximately 10^5 recombinant plaques. DNA clones encoding the 23kD and 38kD antigens could not be detected with these anti-
30 bodies, possibly because the native epitope is modified or topographically complex, or because the

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antigen-antibody interaction is too weak to be recognized by current detection methods.

III. Probing of Arrays of lambda gt11 DNA
Clones with Antibody Probes

05 0.2 ml of a saturated culture of Y1090 was
added to 2.5 ml of molten LB soft agar, poured onto
100 mm plates containing 1.5% LB agar and allowed to
harden at room temperature for 10 min. 100 ul of
10 phage plate stock containing approximately 10^{11}
pfu/ml of the lambda gt11 DNA clones of interest
were placed into alternate wells of 96-well tissue
culture plates. A multi-pronged transfer device was
placed briefly in the wells containing phage and
then touched lightly to the surface of the plate
15 onto which the soft agar had been poured. The
plates were then incubated at 42°C for approximately
3 hours, at which point clear plaques approximately
5mm in diameter were visible. The plates were then
overlaid with nitrocellulose filters saturated with
20 10mM isopropylthiogalactoside (IPTG) and incubated
at 37°C for 3.5 hours. Subsequent processing of
filters for detection of antigen was identical to
the procedures described for screening of lambda
gt11 library with antibody probes.

25 Immunoscreening of the lambda gt11 library to
isolate clones reactive with monoclonal antibodies
specific for the 65kD antigen is described in the
Exemplification.

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IV. Recombinant DNA Manipulation

DNA from recombinant lambda gt11 clones was isolated and mapped with restriction endonucleases by standard techniques. Davis, R.W. et al.,
05 Advanced Bacterial Genetics: A Manual for Genetic Engineering, Cold Spring Harbor (1980).

Figure 1 shows the genomic DNA restriction map deduced for each of the genes encoding the five M. tuberculosis antigens and illustrates how each of
10 the cloned DNAs aligns with that map. All clones isolated with monoclonal antibodies directed against any single antigen align with a single genomic DNA segment. This indicates that all clones were
15 isolated because they express the protein of interest rather than an unrelated polypeptide containing a similar or identical epitope. In addition, this result suggests that each antigen is the product of a single gene.

The orientation of each DNA insert in the
20 recombinant clones was determined by restriction analysis. Only among the clones for the 65kD antigen were the inserts found in both possible orientations relative to the direction of lac Z transcription in lambda gt11. This suggests that
25 this protein can be expressed in E. coli from signals independent of those provided by lac Z. Similar results have been obtained for recombinant DNA clones encoding the 65kD antigens of M. bovis and M. leprae. Thole, J.E.R. et al., Infectious
30 Immunology, 50:800-806 (1985); Young, R.A. et al., Nature, 316:450-452 (1985).

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The nucleotide sequences of three regions of the M. tuberculosis DNA were determined: 1) the region containing the M. tuberculosis 19kD gene; 2) the region containing the M. tuberculosis 71kD gene; and 3) the region containing the 65kD gene. The three sequences are represented in Figures 6-8. Sequences were determined using standard techniques, which are described in the Exemplification.

V. Filter hybridization of Insert DNA

Arrays of lambda gtl1 clones were created as described above and incubated at 42° for 5 hours. The plates were then overlaid with nitrocellulose filters and placed at 4°C for 1 hour. Probe DNA was labelled with ³²P by nick translation. Filter hybridization was performed as described by Davis et al. in Advanced Bacterial Genetics: A Manual for Genetic Engineering, Cold Spring Harbor (1980), the teachings of which are incorporated herein by reference. Hybridization conditions were as follows: 50% v/v formamide, 5x SSPE (1x SSPE is 0.18M NaCl, 10mM Na_{1.5}H_{1.5}PO₄, 1mM Na₂ EDTA, pH 7.0), 1x Denhardt's solution (0.02% w/v Ficoll, 0.02% w/v polyvinylpyrrolidone, 0.02% w/v bovine serum albumin), 0.3% NaDodSO₄ at 42°C for approximately 16 hours, followed by washing in 2x SSPE, 0.2% NaDodSO₄ at 45°C.

VI. Recombinant Antigens Recognized by Rabbit Serum

The response of a second animal to an antigen preparation of M. tuberculosis was assessed by

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examining the reactivity of rabbit anti-M. tuberculosis hyperimmune sera with recombinant antigens. Cloned lambda gt11 recombinants were arrayed on lawns of E. coli and probed with the rabbit hyper-immune serum. Anti-M. tuberculosis hyperimmune serum, produced by repeated immunization of rabbits with M. tuberculosis H37Rv culture filtrate, was provided by J. Bennedsen (Statens Seruminstitut, Copenhagen, Denmark). These sera were used at 1:100 dilution.

These sera produced positive signals with lambda gt11 clones encoding each of the five M. tuberculosis epitopes which had been isolated with murine monoclonal antibodies (Figure 2). Particularly strong signals were observed with the 65kD and 71kD antigens (Figure 2). These results demonstrate that mice and rabbits can mount an antibody response to the same protein antigens of M. tuberculosis.

Clones for the five M. tuberculosis antigens were detected at similar frequencies in the lambda gt11 recombinant DNA library. Thus, the number and type of antigen-producing clones isolated with polyclonal serum antibodies should reflect the relative titer and diversity of the individual antibodies in this serum.

To determine whether any of the 5 M. tuberculosis antigens are relatively immunodominant in the rabbit humoral immune response to M. tuberculosis, the M. tuberculosis lambda gt11 recombinant DNA library was screened with the rabbit serum. Forty signal-producing clones were isolated, arrayed on

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lawns of E. coli Y1090 and probed with monoclonal antibodies directed against each of the 5 recombinant M. tuberculosis protein antigens. Remarkably, 17 of the 40 clones (43%) reacted strongly with at least one of the four anti-65kD monoclonal antibodies tested. An additional six clones (15%) reacted strongly with the anti-17kD monoclonal antibody (IT-11). This indicates that a large proportion of the anti-M. tuberculosis antibody present in the rabbit serum was directed against the 65kD antigen of M. tuberculosis and suggests that it is a dominant antigen for the rabbit humoral immune response. Seventeen of the clones did not react with any of the monoclonal antibodies tested, suggesting that the rabbit sera may identify M. tuberculosis proteins not recognized by the murine antibodies.

VII. Antigenic Relatedness of M. tuberculosis and M. leprae Proteins

There is evidence that M. tuberculosis and M. leprae share immunologically important antigens. To assess this further, an investigation of the exact nature of the immunological relatedness among recombinant protein antigens of M. tuberculosis and M. leprae was conducted.

For each of five M. tuberculosis and four M. leprae protein antigens, a single recombinant DNA clone containing most or all of the gene of interest was used to express antigen in the following manner. The recombinant phage clones were arrayed on a lawn

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of E. coli Y1090, which was then grown and induced for antigen expression.

05 Antigen immobilized on nitrocellulose filters was then probed with 26 individual anti-M. tuberculosis and M. leprae monoclonal antibodies. Figure 3 shows the array of DNA clones used and the results obtained with the anti-M tuberculosis antibodies IT-1, IT-3, IT-11, IT-16, and IT-31, which recognized proteins of 14kD, 12kD, 71kD, 19kD and 65kD
10 respectively. Table 1 details the full results of these cross-screening experiments, showing the reactivity of antigen expressed from individual recombinant DNA clones with each of the individual monoclonal antibodies. Clones were scored as
15 positive only if the signal produced was clearly greater than the background signal produced by the non-recombinant lambda gt11 clone included in each array.

[illegible][illegible]

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Several conclusions can be drawn from the results shown in Table 1. Among the 11 monoclonal antibodies that recognize a 65kD antigen, 7 react with the 65kD protein from both mycobacteria (IT-31, C1.1, IIH9 (identical to IT-33), IIC8, T2.3, Y1-2, SA2.D7C), one antibody reacts only with the M. tuberculosis 65kD protein (IT-13), and two antibodies react only with the M. leprae 65kD protein (IIIE9 and IIIC8). One antibody, ML30A, cross-reacts with an antigen in E. coli and could not specifically identify antigen-producing clones. These results indicate that the 65kD protein antigens of M. tuberculosis and M. leprae are homologues and share a number of epitopes. In addition to these shared epitopes, however, both 65kD antigens have epitopes that are specific for one species relative to the other.

No cross-reactivity was observed between other antigens of these two mycobacterial species. Because monoclonal antibodies recognize a single epitope and because only one or a few antibodies were available for each antigen, it is not clear whether the 65kD proteins are the only homologous protein antigens of M. tuberculosis and M. leprae. Among the antigens for which lambda gt11 clones have been isolated, the 18kD antigen of M. leprae and the 19kD antigen of M. tuberculosis are of similar size. To determine whether these two antigens are related, the homology of the DNA sequences that encode these antigens was examined. At conditions of moderate stringency, no hybridization was observed between

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the insert DNA and Y3147 (an M. tuberculosis 19kD clone) and Y3179 (an M. leprae 18kD clone). This indicates no significant homology between the DNA sequences of the insert DNAs of these two clones. This result suggests that the M. tuberculosis 19kD and the M. leprae 18kD proteins are unlikely to be homologous.

As a result of the work described, recombinant DNA clones encoding five major protein antigens of M. tuberculosis were isolated through the use of an extensive collection of well-characterized murine monoclonal antibodies. These five proteins were also found to be major antigens in the rabbit humoral immune response to M. tuberculosis. One of these antigens, the 65kD protein, is shared with another other mycobacterial pathogen M. leprae.

Several lines of evidence indicate that the 65kD antigen is among the most immunodominant of the protein antigens of M. tuberculosis. Eleven of the 25 different M. tuberculosis and M. leprae monoclonal antibodies examined in this study recognized the 65kD recombinant antigen from one or both mycobacteria. In addition, almost half of the recombinant DNA clones isolated with rabbit polyclonal anti-M. tuberculosis sera express the 65kD antigen, reflecting the predominance of antibody to this antigen in these sera.

Considerable evidence indicates that the 65kD antigen plays an important role in the human response to tuberculosis. Antibodies directed against this protein can be detected in the serum of

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patients with tuberculosis. The 65kD antigen is present in purified protein derivatives (PPD's) of M. tuberculosis, M. bovis, and other mycobacteria. Thole, J.E.R. et al., Infection Immunity, 50:800-806 (1985). Finally, helper T cell clones reactive with recombinant 65kD antigen have been isolated from patients with tuberculosis, indicating that this antigen is involved in the cell-mediated as well as the humoral immune response to tuberculosis.

Among the major antigens of the leprosy bacillus, the 65kD antigen appears to elicit antibody and T cell responses similar to those observed for the M. tuberculosis antigen. Both serum antibodies and T cells directed against the 65kD M. leprae antigen have been observed in patients with leprosy. Britton, W.J. et al., Journal of Immunology, 135:4171-4177 (1985); Mustafa, A.S. et al., Nature, 319:63-66 (1986). In addition, T cell clones from leprosy patients have been found to respond to recombinant 65kD protein of M. bovis, as well as to PPD's from both M. bovis BCG and M. leprae. Emmrich, F. et al., Journal of Experimental Medicine, 163:1024-1029 (1986); Shankar, P. et al., Journal of Immunology, 136:4255-4263 (1986). It is interesting to note that in vaccine trials in Asia and Africa, BCG provided significant protection against leprosy, ranging from 20% to 80%. Fine, P., Tubercle, 65:137-153 (1984). An intriguing possibility is that the M. bovis BCG 65kD antigen is involved in engendering the immune protection

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provided by this vaccine against M. leprae, as well as against M. tuberculosis.

05 In addition to the 65kD antigen, there is evidence that the 19kD and 71kD antigens of M. tuberculosis may be particularly important in the immune response to this bacillus. Helper T cell clones from tuberculosis patients have been isolated which respond to the recombinant 19kD protein. The 71kD antigen is recognized by the humoral immune system of both mice and rabbits, and antibody to this antigen has been shown to be a prominent component of hyperimmune anti-M. tuberculosis rabbit sera.

15 VIII. Isolation of DNA Clones for Genes Encoding Proteins of Additional Mycobacteria

Using the procedures described above for isolation of genes encoding M. tuberculosis proteins, genes encoding proteins of additional mycobacteria were isolated. DNA clones containing genes encoding the following proteins were isolated:

<u>Mycobacterium</u>	<u>Protein</u>	<u>Clone</u>
<u>M. bovis</u> BCG	71kD	PL1-101
	65kD	PL1-105
	19kD	PL1-501
	14kD	PL1-502
<u>M. smegmatis</u>	65kD	PL1-206
<u>M. avium</u>	65kD	PL1-401
<u>M. africanum</u>	65kD	PL1-301

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For purposes of comparison, genes encoding the following proteins were isolated for M. tuberculosis and M. leprae:

	<u>Mycobacterium</u>	<u>Protein</u>	<u>Clone</u>
05	<u>M. tuberculosis</u>	71kD	Y3272
		65kD	Y3150
		19kD	Y3147
		14kD	Y3248
	<u>M. leprae</u>	65kD	

10 The following strains were used for this purpose:

	<u>Species</u>	<u>Isolate</u>
	<u>M. leprae</u>	Armadillo isolate (WHO)
	<u>M. tuberculosis</u>	Erdmann strain
15	<u>M. africanum</u>	African clinical isolate
	<u>M. bovis</u> BCG	Danish vaccine strain
	<u>M. smegmatis</u>	MC ² -6
	<u>M. avium</u>	AIDS patient isolate

20 DNA from recombinant lambda gt11 clones was isolated, as described above, and mapped with restriction endonucleases, using standard techniques. Davis, R.W. et al., Advanced Bacterial Genetics: A Manual for Genetic Engineering, Cold Spring Harbor (1980).

25 Figure 4 presents a comparison of the restriction maps for four genes of M. tuberculosis with the restriction maps for four genes of M. bovis BCG which encode proteins of the same molecular weight. As is evident from the figure, in each case, the

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restriction sites on the two genes (e.g., those on the M. tuberculosis gene and those on the M. bovis gene which encodes a protein of the same molecular weight) are essentially identical. This indicates that the sequence of the genes of the two mycobacteria (at least those encoding these four proteins) are very similar and, therefore, the proteins they encode are also very similar.

Figure 5 presents a comparison of the restriction map for the gene encoding the 65kD protein for the six mycobacteria. As is evident, the restriction maps for the genes encoding the 65kD protein of M. tuberculosis, M. africanum, M. bovis BCG, M. smegmatis and M. avium are essentially identical. The fact that there is no detectable difference among these mycobacteria at the level of the restriction map is an indication that, at least at this level, the encoded proteins are the same.

As is also evident, the map of the M. leprae 65kD gene has several identical restriction sites in common with those of the other mycobacteria; it also has two sites not found in the other genes and lacks three sites present in the others. This indicates that, at the level of the restriction map, there are similarities in the DNA (and the encoded protein). In addition, however, there are differences apparent at this level.

IX. Diagnostic, Therapeutic and Preventive Applications

The isolation of genes encoding major protein antigens of M. tuberculosis makes it possible to

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address problems which presently exist in diagnosing treating and preventing tuberculosis. Isolation of genes encoding proteins of other mycobacteria, such as M. bovis BCG, M. africanum, M. smegmatis and M. avium makes it possible to address similar problems in diseases which they cause.

The nucleotide sequence of three of the five genes has been determined. The sequence of the remaining genes can be determined using well-known methods, such as that of Sanger et al. Sanger, F. et.al., Proceedings of the National Academy of Sciences, USA, 74:5463-5467 (1977). The amino acid sequence of each of the immunodominant proteins has been deduced from the nucleotide sequence of the three genes and can be done for the others.

Identification and characterization of the genes for major tuberculosis protein antigens and of the proteins themselves make it possible to develop improved reagents for diagnosis and immuno-prophylaxis of tuberculosis. Proteins antigens encoded by an entire gene, or amino acid sequences (e.g., peptides, protein fragments) which make up the antigenic determinant of a M. tuberculosis antigen (i.e., M. tuberculosis-specific antigenic determinants) may be used in serodiagnostic tests and skin tests. Such antigens would be highly specific to the tuberculosis bacillus and the tests in which they are used would also be highly specific. Highly specific serological tests would be of great value in screening populations for

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individuals producing antibodies to M. tuberculosis-specific antigenic determinants; in monitoring the development of active disease in individuals and in assessing the efficacy of treatment. As a result, early diagnosis of tuberculosis will be feasible, thus making it possible to institute treatment at an early stage of the disease and, in turn, to reduce the likelihood it will be transmitted.

As a result of the work described, it is also possible to determine which segment(s) of the M. tuberculosis antigen is recognized by M. tuberculosis-specific T cells. A mixture of peptides recognized by helper T cells can serve as a specific skin test antigen useful in assessing immunological status (delayed hypersensitivity) of infected individuals and those with whom they come in contact. This specific skin test antigen would be useful in evaluating rapidly the immunological efficacy of anti-tuberculosis vaccines.

It is reasonable to expect that the products encoded by M. tuberculosis genes, particularly those shown to be recognized by helper T cells, are themselves immunogenic and thus useful components of vaccines against tuberculosis. These products include proteins and portions of such proteins (e.g., polypeptides and peptides). For example, one approach to vaccine development is the introduction of genes encoding products (e.g., polypeptides) which provide immunological protection into viruses such as vaccinia virus, or bacteria, such as cultivatable mycobacteria, thus producing a vaccine

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capable of engendering long-lasting and very specific immunity. The genes encoding five immunodominant protein antigens of the tuberculosis bacillus, described herein, are useful for that
05 purpose; genes encoding the 65kD, 19kD and 71kD antigens, or a portion thereof, are particularly valuable in vaccine construction.

Because of the similarities in the DNA encoding similarly-sized proteins and, thus, of the encoded
10 proteins themselves, it is possible that, for example, a vaccine effective against two or more of the mycobacteria can be produced.

EXEMPLIFICATION

Isolation and Analysis of Recombinants Expressing 15 the 65kD M. tuberculosis Antigen

The recombinant DNA library of M. tuberculosis genomic DNA fragments in the lambda gt11 vector was constructed as described above. Recombinant phage lambda RY3143 and lambda RY3146 were used. Young,
20 R.A. et al., Proceedings of the National Academy of Sciences, USA, 82:2583-2587 (1985). Subclones of the mycobacterial DNA inserts in these recombinant phage were constructed in pUC19 or M13mp9 vectors using standard recombinant DNA techniques. Messing,
25 J. and J. Viera, Gene, 19:269-276 (1982). Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

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Monoclonal antibodies specific for the 65kD antigen were obtained from the Immunology of Tuberculosis Scientific Working Group under a grant from the WHO/World Bank/UNDP Special Program for Vaccine Development. These antibodies included IT-13 (WTB-78), IT-31 (SA2D5H4), and IT-33 (MLIIH9). Coates, A.R.M. et al., Lancet, 2:167-169 (1981). Gillis, T.P. and T.M. Buchanon, Immunology, 37:172-178 (1982). Anti-B-galactosidase antibodies were purchased from CooperBiomedical. Polyclonal rabbit antisera directed against a sonicate of M. tuberculosis strain H37Rv were elicited as described by Minden and co-workers. Minden, P. et al., Infect. Immun., 46:519-525 (1984). Results are shown in Table 2.

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TABLE 2: PATTERNS OF ANTIBODY REACTIVITIES^a

<u>Number of Clones</u>		<u>Reactivity with Antibodies</u>		
		<u>IT-13</u>	<u>IT-31</u>	<u>IT-33</u>
05	27	+	+	+
	1	+	+	+
	2	+	-	+
	3	-	+	+
	1	+	-	-
	2	-	+	-
10	2	-	-	+

^a: Recombinant clones expressing antigens reactive with the 65kD antigen specific monoclonal antibodies IT-13, IT-31, and IT-33 were isolated as described above. For the initial screen, a pool of the three antibodies was used; it contained a 1:1000 dilution of each antibody to screen a total of about 8×10^5 recombinant phage from the lambda gt11-M. tuberculosis library. To determine which monoclonal antibody reacted with which of the 38 plaque-purified recombinants, about 100 pfu of each recombinant phage were inoculated in small spots on a lawn of Y1090. The phage were allowed to grow and induced to synthesize the foreign proteins as described previously. The filters were then reacted with a 1:1000 dilution of one of the monoclonal hybridoma antibodies as described above.

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The lambda gt11-M. tuberculosis library was screened with the monoclonal antibodies specific for the 65kD antigen and clones reactive with them were isolated essentially as described by Young et al.

05 Young, R.A. et al., Proceedings of the National Academy of Sciences, USA, 82:2583-2587 (1985). Briefly, for each 150mm LB plate, 0.6ml of a fresh overnight culture of Y1090 was infected with 1-2

10 $\times 10^5$ plaque forming units of the library. After 3.5-4 hours of growth at 42°C, the plaques were overlaid with a dry nitrocellulose filter which had been saturated with 10mM isopropyl-B-D-thiogalactopyranoside (IPTG). The plates were incubated an

15 additional 3.5-4 hours at 37°C and then removed to room temperature and the position of the filters marked. The filters were washed briefly in TBST (50 mM Tris-HCl, pH 8, 150mM NaCl, 0.05% Tween 20) and then incubated in TBST + 20% fetal calf serum.

20 After 30 minutes at room temperature, the filters were transferred to TBST plus antibody. For the initial screen, the antibody mix contained a 1:1000 dilution of IT-13, IT-31, and IT-33. The filters were incubated with the antibody solution overnight at 4°C with gentle agitation, washed in TBST and

25 reacted with biotinylated goat anti-mouse immunoglobulin, the Vectastain ABC reagent, and developer as described by the manufacturer (Vector Laboratories). After the color had developed the filters were washed with several changes of water

30 and air dried. Phage corresponding to positive signals were twice plaque purified. To determine

-31-

which monoclonal antibodies reacted with which of the recombinant phage, about 100 pfu of a purified phage stock were inoculated in a small spot on a lawn of Y1090 bacteria on an LB plate. The phage were allowed to grow and induced to synthesize the foreign proteins as described above. The filters were then reacted with a 1:1000 dilution of one of the monoclonal antibodies. The subsequent steps were the same as for the initial screen.

Western blot assays were carried out as follows: Cells containing phage or plasmids in which the expression of the foreign sequences was under the control of the E. coli lac gene regulatory sequences were induced to synthesize the foreign proteins by incubating the cells in the presence of 2.5mM IPTG for 2 hours. Crude lysates of cells expressing lambda gt11 recombinants were made as described in Huynh et al. Huynh, T.V. et al., In: DNA Cloning Techniques: A Practical Approach, (D. Glover, ed.) IRL Press, Oxford, Vol. 1, pp. 49-78 (1985). Crude lysates of cells expressing plasmid encoded proteins were made by harvesting cells from overnight cultures and resuspending the cells in 10 mM Tris pH7.5/10 mM EDTA containing 100 ug lysozyme/ml. After 10 minutes at room temperature, SDS was added to a final concentration of 0.5%. A protease inhibitor (Trasylol, Boehringer Mannheim) was added to all crude lysates at a final concentration of 0.3%. The crude protein preparations were electrophoresed on 10% polyacrylamide-SDS Laemmli gels and the separate proteins electrophor-

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etically transferred to nitrocellulose. Laemmli, U.K., Nature, 227:680-685 (1970). Towbin, H. et al., Proceedings of the National Academy of Sciences, USA, 76:4350-4354 (1979). The immobilized proteins were reacted with a 1:1000 dilution of monoclonal antibody IT-13 in TBST overnight at 4°C. The nitrocellulose filters were then washed, reacted with peroxidase-conjugated goat anti-mouse immunoglobulin, and developed as described by Niman and co-workers. Niman, H.L. et al., Proceedings of the National Academy of Sciences, USA, 80:4949-4953 (1983).

The sequences of 5'-end-labeled restriction fragments of the mycobacterial DNA were determined by a modification of the partial chemical degradation technique of Maxam and Gilbert. Brow, M.A.D. et al., Mol. Biol. Evol., 2:1-12 (1985). Maxam, A.M. and W. Gilbert, Proceedings of the National Academy of Sciences, USA, 74:560-564 (1976). For the M13/dideoxy sequencing studies, Sau3AI fragments from the mycobacterial DNA inserts were subcloned into the BamHI site of M13mp9. Phage DNA was isolated from the M13 recombinants and subjected to the dideoxy chain termination sequencing reactions. Biggin, M.D. et al., Proceedings of the National Academy of Sciences, USA, 80:3963-3965 (1983). Sanger, F. et al., Journal of Molecular Biology, 143:161-178 (1980). The products of the sequencing reactions were electrophoresed on 6% acrylamide/7M urea/0.5-2.5 x TBE gradient sequencing gels. The gels were dried

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under vacuum and exposed to Kodak XRP-1 film. The nucleotide sequences were determined independently for both strands of the mycobacterial DNA.

05 Computer-aided analyses of the nucleic acid sequences and deduced protein sequences were performed using the Databases and programs provided by the Nucleic Acid and Protein Identification Resources of the National Institutes of Health as well as the programs of Chou and Fasman and Hopp and
10 Woods. Chou, P.Y. and G.D. Fasman, Adv. Enzym., 47:45-148 (1978). Hopp, T.P. and K.P. Woods, Proceedings of the National Academy of Sciences, USA, 78:3824-3828 (1981). The nucleotide sequence of the region containing the M. tuberculosis 65kD
15 gene and the deduced amino acid sequence of the two long open reading frames are represented in Figure 8.

B-galactosidase assays were also carried out. Cells were grown in LB broth or LB broth plus 2.5mM
20 IPTG to an OD₆₀₀ of about 0.3. Crude lysates were made and b-galactosidase activity assayed as described by Miller. Miller, J.H., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1972).

25 Equivalents

Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents to the specific materials and components described herein. Such
30 equivalents are intended to be encompassed in the scope of the following claims.

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CLAIMS

1. Isolated DNA encoding an immunogenic protein antigen of Mycobacterium tuberculosis.
- 05 2. DNA of Claim 1 selected from the group consisting of DNA encoding Mycobacterium tuberculosis protein antigens of molecular weight 71kD, 65kD, 19kD, 14kD and 12kD.
- 10 3. Isolated DNA encoding an immunodominant protein antigen of Mycobacterium tuberculosis, the protein antigen having a molecular weight of approximately 65kD and recognized by a monoclonal antibody selected from the group consisting of: IT-31; C1.1; IIH9; IIC8; T2.3; Y1-2; SA2.D7C and IT-13.
- 15 4. Isolated DNA encoding an immunodominant protein antigen of Mycobacterium tuberculosis, the protein antigen having a molecular weight of approximately 19kD and recognized by a monoclonal antibody selected from the group consisting of: IT-10; IT-12; IT-16; and IT-19.
- 20 5. Isolated DNA encoding an immunodominant protein antigen of Mycobacterium tuberculosis, the protein antigen having a molecular weight of approximately 71kD and recognized by the monoclonal antibody IT-11.
- 25

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6. Isolated DNA encoding an antigenic determinant of Mycobacterium tuberculosis protein.
- 05 7. DNA of Claim 6 which encodes an antigenic determinant selected from the group consisting of antigenic determinants of Mycobacterium tuberculosis proteins of molecular weight 71kD, 65kD, 19kD, 14kD and 12kD.
- 10 8. Isolated DNA encoding an amino acid sequence of an antigenic determinant of Mycobacterium tuberculosis protein, said protein having a molecular weight of approximately 65kD.
- 15 9. Isolated Mycobacterium tuberculosis DNA encoding an immunodominant protein antigen having a molecular weight of approximately 65kD, said DNA selected from the group consisting of:
 - a. the DNA insert of clone Y3141;
 - b. the DNA insert of clone Y3143;
 - c. the DNA insert of clone Y3150;
 - d. the DNA insert of clone Y3253; and
 - 20 e. the DNA insert of clone Y3262.
10. A protein antigen encoded by DNA of Claim 9.
- 25 11. A protein antigen of Claim 10, wherein the protein antigen is recognized by a monoclonal antibody selected from the group consisting of IT-31; C1.1; IIH9; IIC8; T2.3; Y1-2; SA2.D7C and IT-13.

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- 05 12. Isolated DNA having a nucleotide sequence selected from the group consisting of: a) the nucleotide sequence represented in Figure 6, or a portion thereof; b) the nucleotide sequence represented in Figure 7, or a portion thereof; and c) the nucleotide sequence represented in Figure 8, or a portion thereof.
- 10 13. A protein or a peptide selected from the group consisting of: a) proteins or peptides encoded by the nucleotide sequence represented in Figure 6, or a portion thereof; b) proteins or peptides encoded by the nucleotide sequence represented in Figure 7, or a portion thereof; and c) proteins or peptides encoded by the nucleotide sequence represented in Figure 8, or a portion thereof.
- 15 14. A peptide having the amino acid sequence of an antigenic determinant of Mycobacterium tuberculosis protein, said antigenic determinant being unique to Mycobacterium tuberculosis protein.
- 20 15. A peptide encoded by isolated Mycobacterium tuberculosis DNA, said peptide recognized by helper T cells.
- 25 16. A peptide encoded by the Mycobacterium tuberculosis DNA insert of clone Y3150 or a portion of said DNA insert.

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17. Isolated DNA encoding a protein of Myco-
bacterium africanum the protein having a
molecular weight of 65kD.
18. Isolated DNA encoding a protein of Myco-
05 bacterium avium, the protein having a molecular
weight of 65kD.
19. A vaccine comprising DNA encoding Mycobacterium
tuberculosis protein in a recombinant vaccine
vector capable of expressing said DNA.
- 10 20. A vaccine of Claim 19 in which the recombinant
vaccine vector is vaccinia virus or cultivat-
able mycobacteria.
21. A vaccine of Claim 20 in which the DNA encodes
the 65kD Mycobacterium tuberculosis protein
15 recognized by the monoclonal antibody IT-13, or
a portion of said protein.
22. A vaccine comprising DNA encoding an antigenic
determinant unique to Mycobacterium tubercu-
losis cultivatable mycobacteria capable of
20 expressing said DNA.
23. A method of detecting antibody against Myco-
bacterium tuberculosis in a biological fluid,
comprising the steps of:
 - a) incubating an immunoabsorbent com-
25 prising a solid phase to which is attached

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immunodeterminant Mycobacterium tuberculosis protein with a sample of the biological fluid to be tested, under conditions which allow the anti-Mycobacterium tuberculosis antibody in the sample to bind to the immunoabsorbent;

b) separating the immunoabsorbent from the sample; and

c) determining if antibody is bound to the immunoabsorbent, as an indication of anti-Mycobacterium tuberculosis in the sample.

24. A method of Claim 23 in which the Mycobacterium tuberculosis protein attached to the solid phase has a molecular weight of approximately 65kD.

25. A method of detecting antibody against Myco-bacterium tuberculosis in a biological fluid, comprising the steps of:

a) incubating an immunoabsorbent comprising a solid phase to which is attached a peptide having the amino acid sequence of an antigenic determinant of Mycobacterium tuberculosis protein with a sample of the biological fluid to be tested, under conditions which allow antibody against Mycobacterium tuberculosis to bind to the immunoabsorbent;

b) separating the immunoabsorbent; and

c) determining if antibody is bound to the immunoabsorbent, as an indication of the

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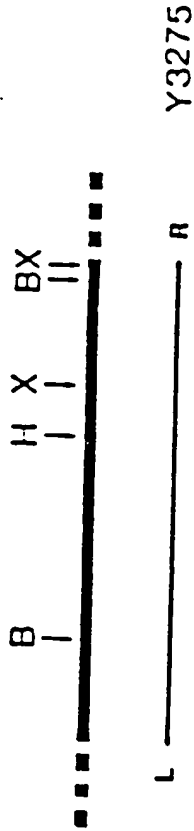
presence of the antibody against Mycobacterium tuberculosis in the sample.

- 05 26. A method of Claim 25 in which the peptide has the amino acid sequence of an antigenic determinant which is unique to Mycobacterium tuberculosis protein.
- 10 27. A kit useful in detecting antibody against Mycobacterium tuberculosis in a biological fluid, comprising a collection of reagents for immunoassay of said antibody, said collection of reagents a solid phase to which is attached immunodeterminant Mycobacterium tuberculosis protein or a peptide having the amino acid sequence of an antigenic determinant of
- 15 Mycobacterium tuberculosis.

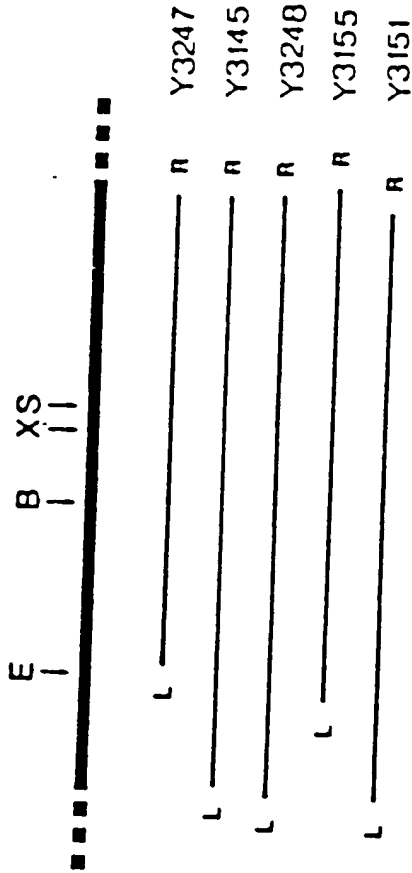
FIGURE 1

1Kb

12K



14K



19K

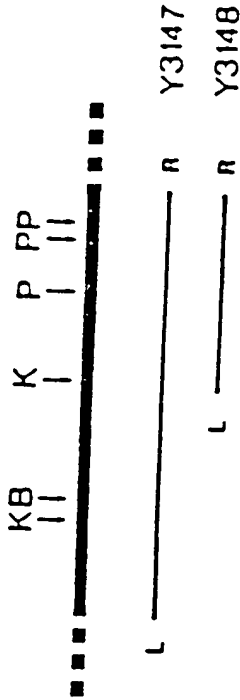
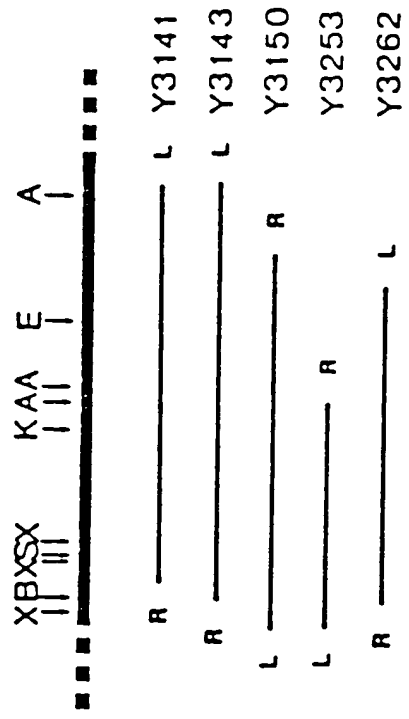


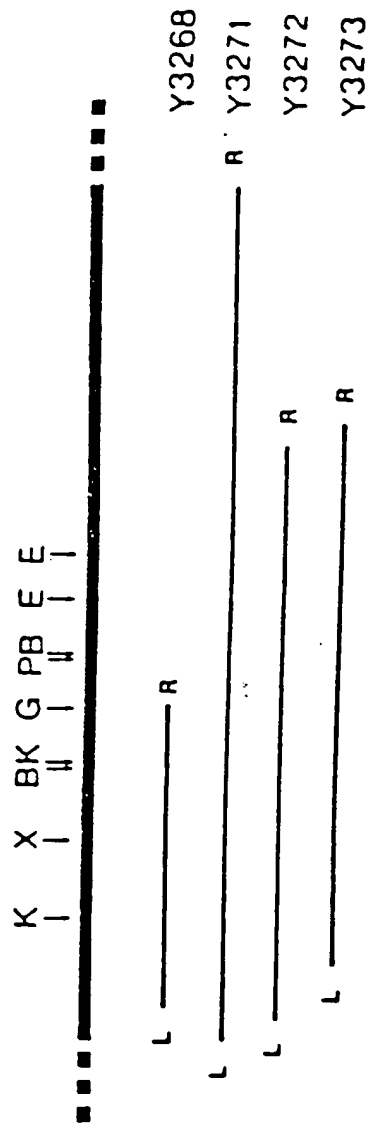
FIGURE 1 (Cont'd)

1Kb

65 K

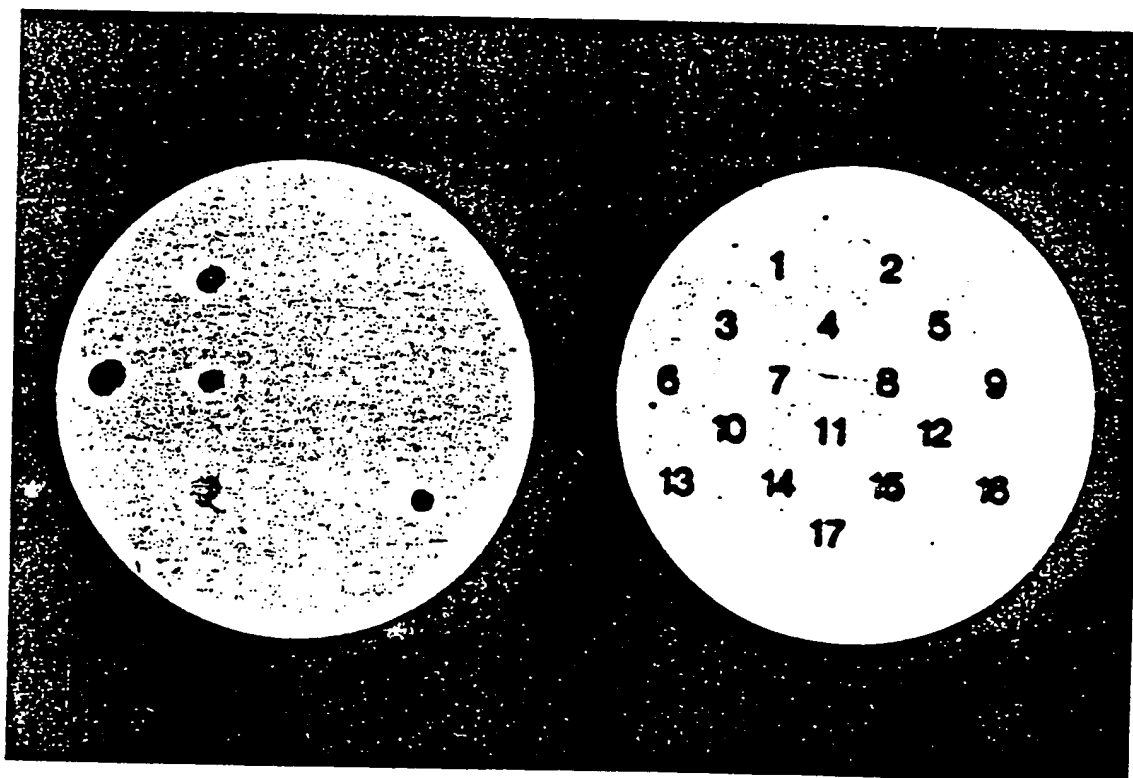


71 K



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FIG.2



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FIG.3

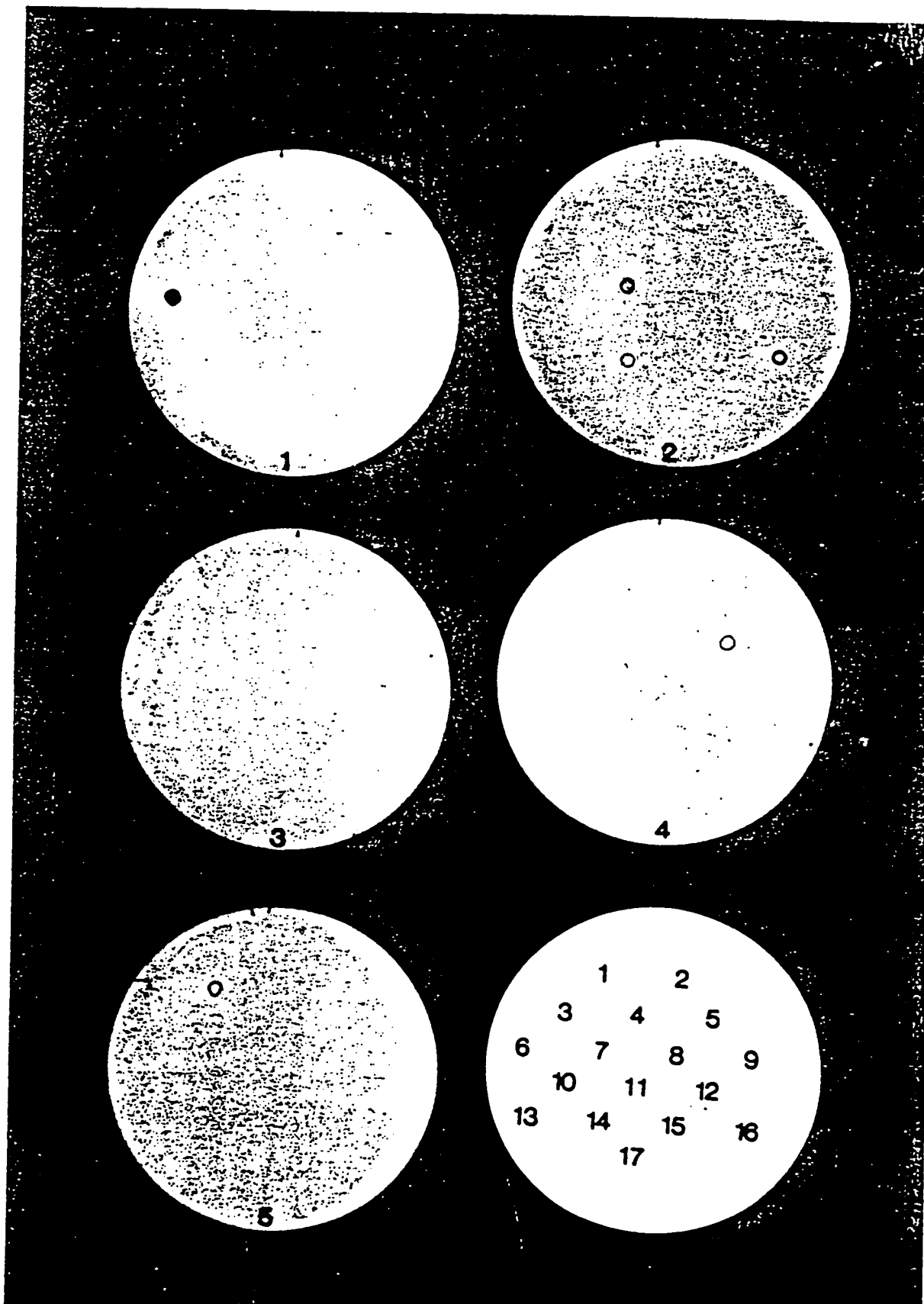
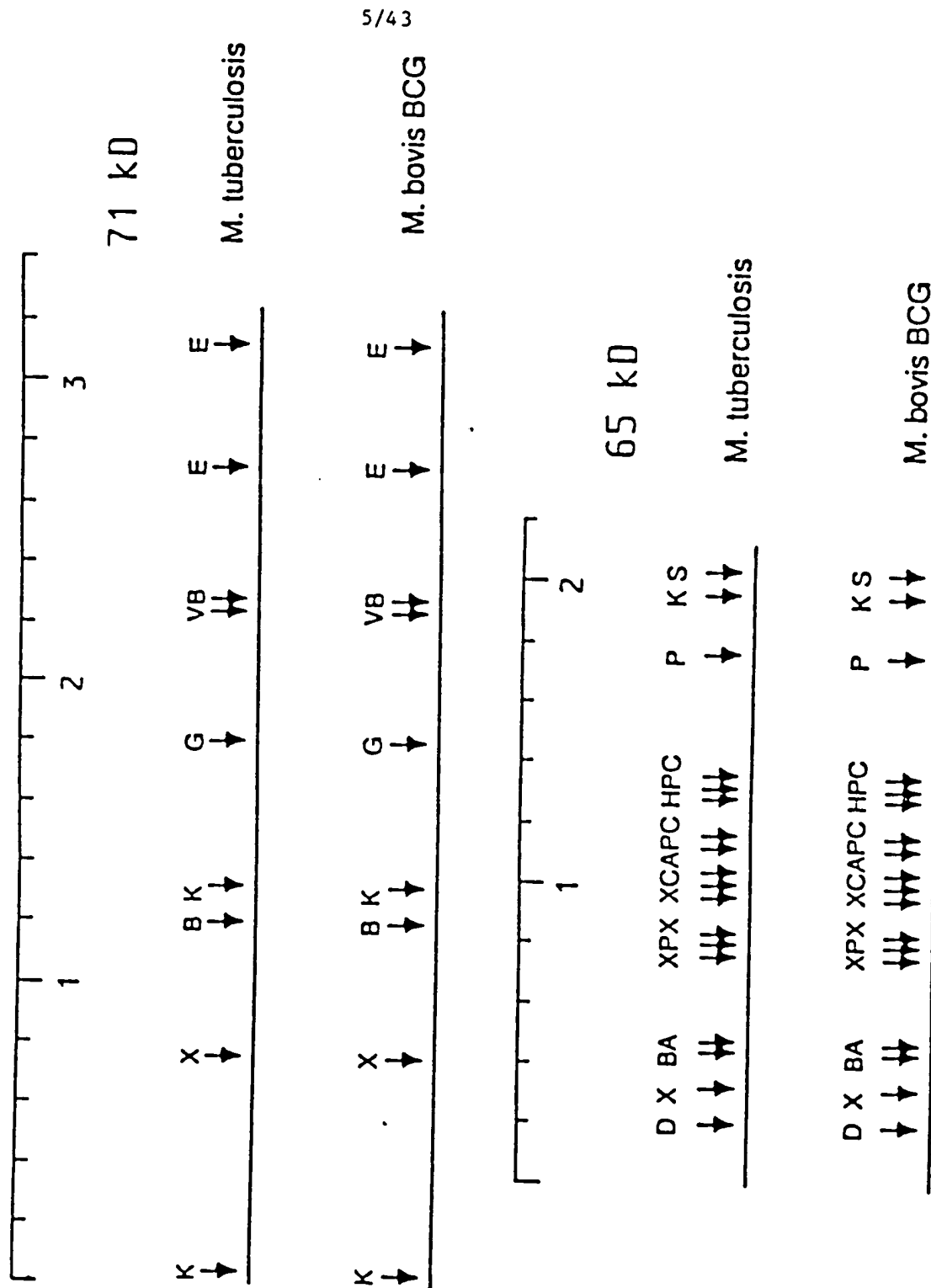
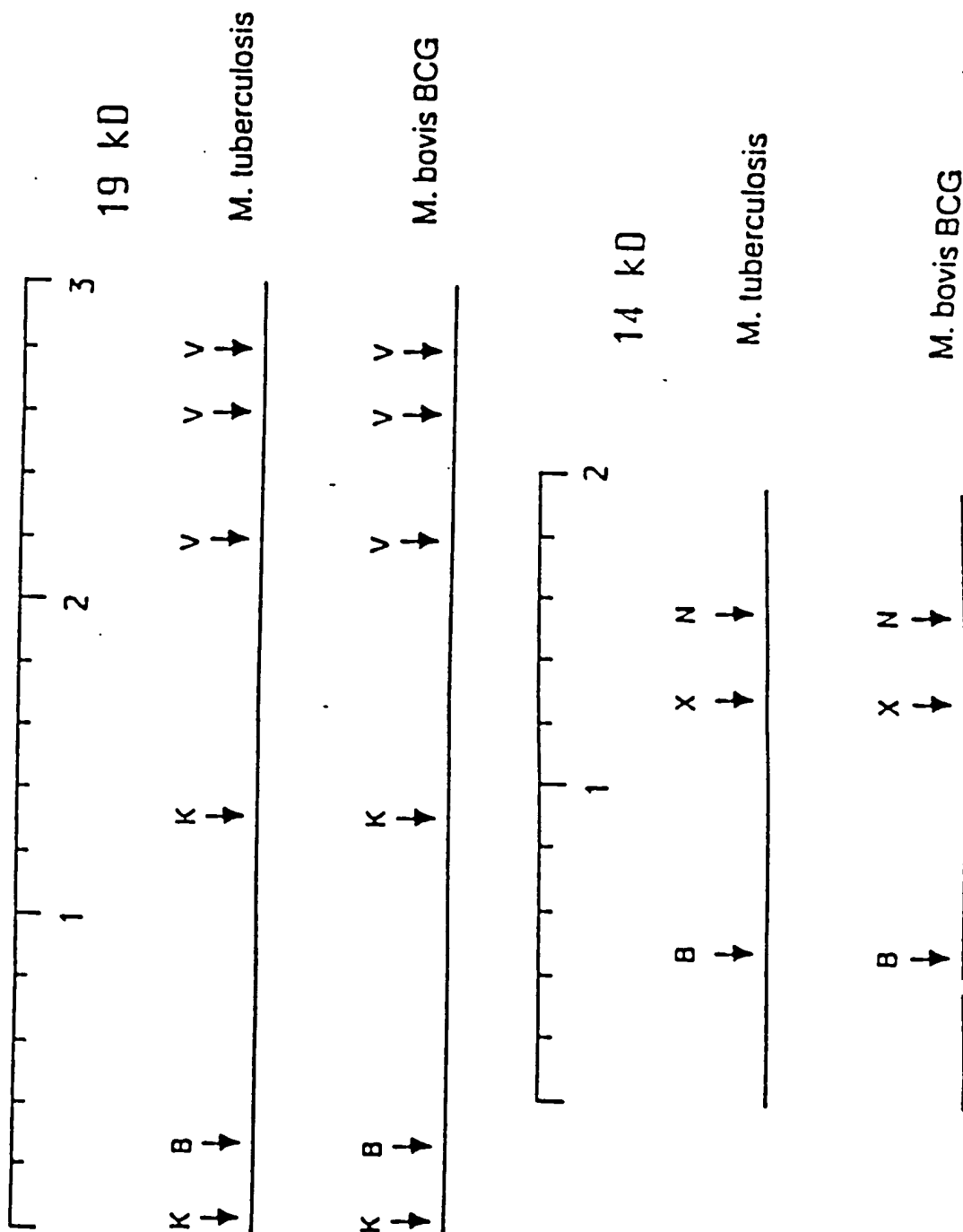


FIGURE 4



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FIGURE 4 (Cont'd)



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FIGURE 6

F R V N R L G E I A R P G A R I A H Q G
S V S I A S V R * P D Q A R G S R T K A
P C Q S P R * D S P T R R A D R A P R R
TTCCGTGTCATCGCCTCGGTGAGATAGCCGACCGAGCGCGGATCGCGCACCAAGGC
10 20 30 40 50 60
AAGGCACAGTTAGCGGAGCCACTCTATCGGGCTGGTCCGCGCCTAGCGGTTCG
E T D I A E T L Y G S W A R P D R V L A
G H * D G R H S L G V L R A S R A G L R
R T L R R P S I A R G P A R I A C W P A

A A Q G Q Q V V Q V M R G V F G H A Q R
P R R V S R L C R L C V A F S A M H N A
R A G S A G C A G Y A W R F R P C T T R
GCCGCGAGGTCAGCAGGTTGTGCAGGTTATGCGTGCGGTTTCGGCCATGCACAACGC
70 80 90 100 110 120
CGGCGCGTCCCAGTCCCAACACAGTCCCAATACGCACCGCAAAAGCCGTACGTGTGCG
G R L T L L N H L N H T A N E A M C L A
R A P D A P Q A P * A H R K R G H V V R
A C P * C T T C T I R P T K P W A C R A

FIGURE 6 (CONT'D)

A E A R E I E V H L R R G L G A R G H L
 R K R G K * K C I S A G A S V P G A I W
 G S A G N R S A S P Q G P R C P G P S G
 GCGAAGCGGGAAATAGAGTGCACTCCGACGGGGCCCTCGGTGCCCGGGGCCATCTG
 130 140 150 160 170 180
 CGCCCTCGGCGCCCTTATCTTACGTAGAGCGTCCCGGAGCCACGGGCCCGGTAGAC
 R F R P F Y F H M E A P A E T G P A M Q
 P L A P F L L A D G C P G R H G P G D P
 S A R S I S T C R R L P R P A R P W R S
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 E L D L H P V D G V G L P G L G D V D R
 N S I S T P S M V W V S P V S V M S T V
 T R S P P R R W C G S P R S R * C R P S
 GAACTCGATCTCCACCCCGTCGATGGTGTGGTCTCCCGGTCTCGGTGATGTCGACCGT
 190 200 210 220 230 240
 CTGAGCTAGAGTGGGCAGCTACACACCCAGAGGGCCAGAGCCACTACAGCTGGCA
 F E I E V G D I T H T E G T E T I D V T
 V R D G G R R H H P D G R D R H R G D
 S S R W G T S P T P R G P R P S T S R R

FIGURE 6 (CONT'D)

R H D E R N L T G R Q C L P E A A A D V
 G T T S E T S P V D S V C P R P Q P T C
 A R R A K P H R S T V S A R G R S R R A
 CGGACGAGCGAAACCTCACCGGTGACAGTGTCTGCCCCGAGGCCGACCGACGTG
 250 260 270 280 290 300
 GCCGTGCTCGCTTTGGAGTGGCCAGCTGTACAGACGGGCTCCGGCTCGGCTGCAC
 P V V L S V E G T S L T Q G L G C G V H
 A R R A F G * R D V T D A R P R L R R A
 C S S R F R V P R C H R G S A A S T G
 10/43
 P P E T A R Q H G A V H V A R T A H H R
 P R R P R A N T V P Y M * P A R R I I A
 P G D R A P T R C R T C S P H G A S S P
 CCCCCGAGACCGCGCCCAACACGGTGCCGTACATGTAGCCCCGACGGCGCATCATCGC
 310 320 330 340 350 360
 GGGGGCCTCTGGCGCGGTTGTGCCACGGCATGTACATCGGGCGTGGCGGTAGTAGCG
 G R L G R A L V T G Y M Y G A R R M M A
 G P S R A G V R H R V H L G C P A D D G
 G S V A R W C P A T C T A R V A C * R R

FIGURE 6 (CONT'D)

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R A G V D V F L H G V R G E P L R R Q H
E P A * M F S C T A C A V N P S G A S T
S R R R C F P A R R A R * T P P A P A P
CGAGCCGGGTAGATGTTTCTGCACGGCGTGCGGTGAACCCCTCCGGCGCAGCAC
370      380      390      400      410      420
GCTCGGCCGATCTACAAAGACGTCCGCACGCGCCACTTGGGAGGCCGCGGTCGTG
S G A Y I N E Q V A H A T F G E P A L V
L R R L H K G A R R A R H V G G A G A G
A P T S T K R C P T R P S G R R R W C R
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R H L S R V H V G L G G D A E H P T E M
A T F P A S T S A W V V T P S T P P K *
P P F P R P R P G W * R R A P H R N D
CGCACCTTCCCGTCACGTCGGCCTGGTGTGACGCCGAGCACCCCGAAATG
430      440      450      460      470      480
GCGGTGGAAGGCGCAGGTGCAGCCGGACCCACCACCTGCGGCTCGTGGGTGCTTAC
A V K G A D V D A Q T T V G L V G G F H
G G K G R G R R G P H H R R A G W R F S
W R E R T W T P R P P S A S C G V S I I

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FIGURE 6 (CONT'D)

I D M A V G V D D R D H G A V G S A V G
 S T W L W V * M T A T T G R S A P R W A
 R H G C G C R * P R P R G G R L R G G R
 ATCGACATGGCTGTGGTGTAGATACCGACCGAGGGGGTCCGGTGGG
 490 500 510 520 530 540
 TAGCTGTACCGACACCATCTACTGGCTGGTCCCGCAGCGGCGCCACCG
 D V H S H T Y I V A V V P R D A G R H A
 R C P Q P H L H G R G R P P R S R P P R
 S M A T P T S S R S W P A T P E A T P A
 12/43
 A I Q V Q R G G G H L G G H Q R V D D D
 R Y K S S A A A T S V D T N G S M T I
 D T S P A R R R P P R W T P T G R * R S
 GCGATACAAGTCCAGCGGGCGGCCACCTCGGTGGACACCAACGGTGGATGACGAT
 550 560 570 580 590 600
 CGCTATGTTTCAGGTCGGCGCGCGGTGGAGCCACCTGTGGTGGCCAGCTACTGCTA
 R Y L D L A A A A V E T S V L P D I V I
 S V L G A R R R G G R H V G V P R H R D
 I C T W R P P P W R P P C W R T S S S *

FIGURE 6 (CONT'D)

Q P S V T L N E A D I G D I E S A D L I
 S P V S P S T K L I L E I S N P R T * *
 A Q C H P Q R S * Y W R Y R I R G P D R
 CAGCCAGTGTCAACCCTCAACGAAGCTGATATTGGAGATATCGAATCCGGACCTGATA
 610 620 630 640 650 660
 GTCGGGTCACAGTGGGAGTTGCTTCGACTATAACCTCTATAGCTTAGCGCCTGGACTAT
 L G T D G E V F S I N S I D F G R V Q Y
 A W H * G * R L Q Y Q L Y R I R P G S L
 G L T V R L S A S I P S I S D A S R I S
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 D A R H H L V E A L F R G Q L G L P P Q
 M P G T T W * R P C F A V S W D C R H R
 C P A P P G R G P V S R S A G I A A T G
 GATGCCCGCACCACTGGTAGAGGCCCTGTTCGCGGTGAGCTGGGATTGCCGCCACAG
 670 680 690 700 710 720
 CTACGGGCGGTGGACCATCTCCGGGACAAAGCGCCAGTCGACCTAACGGCGGTGC
 I G P V V Q Y L G Q K A T L Q S Q R W L
 H G A G G P L P G T E R D A P I A A V P

FIGURE 6 (CONT'D)

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A R C W R T S A R N R P * S P N G G C A
A G M H R C R R G T V E K R V R V V P
L G C T D V G A A P S R N E Y A S L S H
W D A P M S A R H R R E T S T R R C P T
GCTGGATGCACCGATGTCGGCGGCACCGTCGAGAAACGAGTACGCGTGTGTCCTCA
730      740      750      760      770      780
CGACCTACGTGGCTACAGCCGCGCGTGGCAGCTCTTGTCTCATGCGCAGCAACAGGGT
S P H V S T P A A G D L F S Y A D N D W
Q S A G I D A R C R R S V L V R R Q G V
P I C R H R R P V T S F R T R T T G C14/43

H H A T I G S L D H T R G Q R G N E S A
T T R P S A A L I T H G D S A A M N P R
P R D H R Q P * S H T G T A R Q * I R D
CACCAGCACCATCGGCAGCCTTGATCACACGCGGGACAGCGCGCAATGAATCCGCG
790      800      810      820      830      840
GTGGTGGCTGGTAGCCGCGGAAC TAGTGTCGCCCTGTGCGCGCGTTACTTAGGCGC
V V R G D A A K I V C P S L A A I F G R
G R S W R C G Q D C V P V A R C H I R S
W A V M P L R S * V R P C R P L S D A I

```

FIGURE 6 (CONT'D)

I G V V E I R C V M Q R * R V F T V C R
 S A S S K S V V S C N G N E C S P C A A
 R R R R N P L C H A T V T S V H R V P P
 ATCGCGTCGTAATCCGTTGTGTCATGCAACGGTAACGAGTGTTCACCGTGTGCCGC
 850 860 870 880 890 900
 TAGCCGACAGCCTTTAGGCAACACAGTACGTTGCCATTGCTCAAGTGGCACACGGCG
 D A D D F D T T D H L P L S H E G H A A
 R R R R F G N H * A V T V L T * R T G G
 P T T S I R Q T M C R Y R T N V T H R R
 15/43
 L D D G S G R F V F H R H Y I A T T V
 W M T A V G G L C S I G T T L P L L R C
 G * R Q W E V C V P S A L H C H Y Y G A
 CTGATGACGGCAGTGGAGGTTGTGTTCCATCGGCACTACATTGCCACTACTACGGTG
 910 920 930 940 950 960
 GACCTACTGCCGTACCCCTCCAACACAAGGTAGCCGTGATGTAACGGTATGATGCCAC
 Q I V A T P P K H E M P V V N G S S R H
 P H R C H S T Q T G D A S C Q W * * P A
 S S P L P L N T N W R C * M A V V V T C

FIGURE 6 (CONT'D)

H A G R C R W R T T L P T R K R E F S A
 T P V D A V G E P R Y R P E R E N F P P
 R R * M P L A N H A T D Q K E R I F R R
 CAGCCGATAGTCGCGTGGCGAACCAACGCTACCGACCAAGAGAGAATTTCGCC
 970 980 990 1000 1010 1020
 GTGCGCCATCTACGGCAACCGCTTGGTGGATGGTCTTCTCTCTTAAAGCGG
 V G T S A T P S G R * R G S L S F K G G
 R R Y I G N A F W A V S W F S L I K R R
 A P L H R Q R V V S G V L F L S N E A A
 16/43
 A P R P R A L L T R I L P K R S S M P M
 H L D L G P C * R A Y C R S G P Q C R W
 T * T S G P A N A H T A E A V L N A D G
 GCACCTAGACCTCGGGCCCTGCTAACGCGCATACTGCCGAGCGGTCCCTCAATGCCGATG
 1030 1040 1050 1060 1070 1080
 CGTGGATCTGGAGCCCGGACGATTGCGCGTATGACGGCTTCGCCAGGAGTTACGGCTAC
 C R S R P G Q * R A Y Q R L P G * H R H

FIGURE 6 (CONT'D)

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V * V E P G A L A C V A S A T R L A S P
 G L G R A R S V R M S G F R D E I G I S
 D R Y D R Q R S T G * S V D * R S R * P
 T A T T G K G A Q G E A W T D G R G S R
 P L R Q A K E H R \sqrt{V} K R G L T V A V A G
 GACCGTACGACAGGCAAGGACAGGCGTGAAGCGTGGACTGACGGTCGGGTAGCCG
 1090 1100 1110 1120 1130 1140
 CTGGCGATGCTCCGTTTCTCGTGTCCTCCACTTCGCACCTGACTGCCAGCGCATCGGC
 V A V V P L P A C P S A H V S P R P L R
 G S R C A F S C L T F R P S V T A T A P
 R * S L C L L V P H L T S Q R D R Y G S
 E P P F W S Q V F P D V Q A T S R L Q E
 S R H S G R R S F R M F K Q Q V D Y R K
 A A I L V A G L S G C S S N K S T T G S
 GAGCGCCATTCTGTGCGAGGTCTTCCGGATGTTCAAGCAACAAGTCGACTACAGGAA
 1150 1160 1170 1180 1190 1200
 CTCGGCGTAAGACCGGTCCAGAAAGCCCTACAAGTTCGTTGTTGATGATGTCCTT
 L R W E P R L D K R I N L C C T S * L F
 A A M R T A P R E P H E L L L D V V P L
 G G N Q D C T K G S T * A V L R S C S A

FIGURE 6 (CONT'D)

A V R P R P R Q A R R Q A P A P P P G R
 R * D H D R G R H D G K P R R R L R A E
 G E T T A A G T T A S P G A A S G P K
 GCGGTGAGACCAGCGCGGACGACGAGCGCAAGCCCGCGCGCTCCGGGCCGA
 1210 1220 1230 1240 1250 1260
 CGCCACTCTGGTGGCGCGTCCGTGCTCCGTTTCGGGGCGCGGAGGCCGGCT
 R H S W S R P L C S P L G R R R A S
 P S V V V A A P V V A L G P A A E P G F
 T L G R G R C A R R C A G A G G P R L
 18/43
 R S S S T V R T R T S P A P W C A Q P R
 G R H R R * G P E R H R L R G V H N R G
 V V I D G K D Q N V T G S V V C T T A A
 AGGTCGTCATCGAGGTAAGGACCAGAACGTCACCGGCTCCGTGGTGCACACCGCGG
 1270 1280 1290 1300 1310 1320
 TCCAGCAGTAGTGCCATTCTGGTCTTGCAAGTGGCGGAGGACACACGTGTGGCGCC
 P R * R R Y P G S R * R S R P T C L R P
 T T M S P L S W F T V P E T T H V V A A
 D D D V T L V L V D G A G H H A C G R G

FIGURE 6 (CONT'D)

P A M S T S R S A G R R P A L P P C S P
 R Q C Q H R D R R G G D R H C R R A H R
 G N V N I A I G G A A T G I A A V L T D
 CCGGCAATGTCACATCGCGATCGGGGGGGCGGACCGGCATTGCCCGCTGCACCG
 1330 1340 1350 1360 1370 1380
 GGCCGTACAGTTGACGCTAGCCGCCCGCGCTGGCCGTAACGGCGCAGTGCC
 R C H * C R S R R P P S R C Q R R A * R
 P L T L M A I P P A A V P M A A T S V S
 A I D V D R D A P R R G A N G G H E G V
 T A T L R R * S P L G S V T S T A S R W
 R Q P S G G E V R W A R * R Q R R H A G
 G N P P E V K S V G L G N V N G V T L G
 ACGGCAACCCTCCGGAGGTGAAGTCCGTTGGGCTCGGTAACGTCAACGGCGTCACGCTGG
 1390 1400 1410 1420 1430 1440
 TGCCGTTGGAGGCCCTCCACTTCAGGCAACCCGAGCCATTGCAGTTGCCGAGTGGGACC
 R C G E P P S T R Q A R Y R * R R * A P
 P L G G S T F D T P S P L T L P T V S P
 A V R R L H L G N P E T V D V A D R Q S

FIGURE 6 (CONT'D)

D T R R A P D R V T P R Q P R T A A T T
 I H V G H R T G * R L G N Q G R Q P L Q
 Y T S G T G Q G N A S A T K D G S H Y K
 GATACAGTCGGCACCAGGTAACGCCCTCGGCAACCAAGGCGCAGCCACTACA
 1450 1460 1470 1480 1490 1500
 CTATGTGACCCCGTGGCCTGTCCCATGCGGAGCCGTTGTTCTCTGCCGTCGGTGATGT
 I C T P C R V P Y R R P L W P R C G S C
 Y V D P V P C P L A E A V L S P L W * L
 V R R A G S L T V G R C G L V A A V V L
 20/43
 R S L G P L P G S T W P T R C H R * T S
 D H W D R Y R G R H G Q P D V T G E Q V
 I T G T A T G V D M A N P M S P V N K S
 AGATCACTGGGACCGCTACCGGGGTCGACATGGCCAAACCCGATGTCACCGGTGAACAAGT
 1510 1520 1530 1540 1550 1560
 TCTAGTGACCCCTGGCGATGGCCCCAGCTGTACCGGTTGGGCTACAGTGGCCACTGTTC
 S * Q S R * R P R C P W G S T V P S C T
 I V P V A V P T S M A L G I D G T F L D
 D S P G S G P D V H G V R H * R H V L R

FIGURE 6 (CONT'D)

R S K S R * P V P N L K R V D A G C E Q
V R N R G D L F L T * S V S M R A V N S
F E I E V T C S * P K A C R C G L * T A
CGTTCGAAATCGAGGTGACCTGTTCTTAACCTAAAGCGTGCATGCGGGCTGTGAACAG
1570 1580 1590 1600 1610 1620
GCAAGCTTTAGCTCCACTGGACAAGGATTGGATTTCGCACAGCTACGCCCGACACTTGTC
T R F R P S R N R V * L T D I R A T F L
N S I S T V Q E * G L A H R H P S H V A
E F D L H G T G L R F R T S A P Q S C R

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R V G A G Q S G L A R R R F E R L P S V
A S E P G S Q A * R G D D S S G C H P S
R R S R A V R P S A A T I R A V A I R Q
CGGTGCGGAGCCGGCAGTCAGCCTAGCGCGGCGACGATTTCGAGCGGTGCCATCCGTC
1630 1640 1650 1660 1670 1680
GCGCAGCCTCGGCCCGTCAGTCCGGATCGCGCCGCTGCTAAGCTCGCCAACGGTAGGCAG
A D S G P L * A * R P S S E L P Q W G D
R R L R A T L G L A A V I R A T A M R *
T P A P C D P R A R R R N S R N G D T L

FIGURE 6 (CONT'D)

K W Q P H R K L G I S G * A T H G D R S
 S G N R T A N S V Y P G E L L T V I V P
 V A T A P Q T R Y I R V S Y S R * S F F
 AAGTGGCAACCGCACCGCAAACTCGGTATATCCGGGTGAGCTACTCACGGTGATCGTTCC
 1690 1700 1710 1720 1730 1740
 TTCACCGTTGGCGTGTGAGCCATATAGGCCCACTCGATGAGTGCCACTAGCAAGG
 L P L R V A F E T Y G P S S S V T I T G
 T A V A G C V R Y I R T L * E R H D N R
 H C G C R L S P I D P H A V * P S R E T
 22/43
 V V R L D H S G D D R Q A E P G A T G L
 L C A L T A E T I A R P S P V L P A W
 C A P * P Q R R R S P G R A R C Y R L G
 GTGTGGCCTTGACCACAGCGGAGACGATCGCCAGGCCGAGCCCGGTGCTACCGGCTTG
 1750 1760 1770 1780 1790 1800
 CAACACGGGAACGTGTGCGCCTCTGCTAGCGGTCCGGCTCGGGCCACGATGGCCGAAC
 N H A K V V A S V I A L G L G T S G A Q
 Q A G Q G C R L R D G P R A R H * R S P
 T R R S W L P S S R W A S G P A V P K A

FIGURE 6 (CONT'D)

A G P * R I A A G E P L E N L G L Q R G
 R D R D V S P R A N R S K T S D C S A A
 G T V T Y R R G R T A R K P R T A A R P
 GCGGACCGTGACGTATCGCCGGCGGAACCGCTCGAAACCTCGGACTGCAGCGCGG
 1810 1820 1830 1840 1850 1860
 CGCCCTGGCACTGACATAGCGGCCCGCTTGGGAGCTTTGGAGCCTGACGTGCGCGCG
 R S R S T D G R A F R E F V E S Q L A A
 P V T V Y R R P R V A R F G R V A A R G
 P G H R I A A P S G S S F R P S C R P R
 23/43
 R N T R P I V D H L Q H D V R R P G A Q
 G I P G P L S I T C S T T C V G P V L K
 E Y P A H C R S P A A R R A S A R C S S
 CGGAATACCGGCCCATTTGTCGATCACCTGCAGCACGACGTGCGTGGCCCGGTCTCAA
 1870 1880 1890 1900 1910 1920
 GCCTTATGGCCGGGTAAACAGCTAGTGGACGTGCTGCACGCGCGGCCACGAGTT
 P I G P G N D I V Q L V V H T P G T S L
 S Y G A W Q R D G A A R R A D A R H E L
 F V R G M T S * R C C S T R R G P A * A

FIGURE 6 (CONT'D)

A R R H D R A G T G V H A G V G Q Q V G
 R V V T I V P G P V C T R A L A S R L V
 A S S R S C R D R C A R G R W P A G W S
 GCGGTCGATCGTGCCGGGACCGGTGTCACGCGGGCGTTGGCCAGCAGGTTGGT
 1930 1940 1950 1960 1970 1980
 CGCGCAGCAGTGTAGCAGGCCCTGGCCACACGTGCGCCCGCAACCGGTGTCACCA
 R T T V I T G P G T H V R A N A L L N T
 A D D R D H R S R H A R P R Q G A P Q D
 R R * S R A P V P T C A P T P W C T P *
 24/43
 H H L V Q P C R I T R D D H R F G G Q V
 T T W C N R A A S P G M T T G S G G R S
 P P G A T V P H H P G * P P V R G A G R
 CACCACCTGGTCAACCGTGCCGCATCACCCGGGATGACCACCGGTTCGGGGGCGAGGTC
 1990 2000 2010 2020 2030 2040
 GTGGTGACACGTTGGCAGCGCGTAGTGGGCCCTACTGGTGCCCAAGCCCCCGTCCAG
 V V Q H L R A A D G P I V V P E P L D
 G G P A V T G C * G P H G G T R P A P R
 W R T C G H R M V R S S W R N P P C T S

FIGURE 6 (CONT'D)

E R P L V I W S G N M S V A D R V N R K
 S A H W * S G P A T * A S L T A S T A S
 A P T G D L V R Q H E R R * P R Q P Q A
 GAGCGCCACTGGTGATCTGGTCCGGCAACATGAGCGTCGCTGACCGCGTCAACCGCAAG
 2050 2060 2070 2080 2090 2100
 CTCGGGGTGACCACTAGACCAGGCCGTTGTACTCGCAGCGACTGGCGCAGTTGGCGTTC
 L A W Q H D P G A V H A D S V A D V A L
 A G V P S R T R C C S R R Q G R * G C A
 R G S T I Q D P L M L T A S R T L R L G
 25/43
 P R H V H R S A F Q R P P R V Q T R Q Q
 R D M S T G P R S S G R P E S R R A S S
 A T C P P V R V P A A A P S P D A P A A
 CCGGACATGTCACCGGTCGCGTTCCAGCGGCCGCCGAGTCCAGACGCCAGCAG
 2110 2120 2130 2140 2150 2160
 GCGCTGTACAGGTGGCCAGGCGCAAGTCCCGCGGGGCTCAGGTCTGCGCGGTGTC
 R S M D V P G R E L P R G S D L R A L L
 A V H G G T R T G A A A G L G S A G A A
 R C T W R D A N W R G G R T W V R W C C

FIGURE 6 (CONT'D)

Q Q V L D E T C Y P L G L R C H P A H R
 S R S S T R R V I R S V S D A T R L I A
 A G P R R D V L S A R S P M P P G S S R
 CAGCAGGTCCTCGACGAGCGTGTATCCGCTCGGTCCTCCGATGCCACCCGGCTCATCGC
 2170 2180 2190 2200 2210 2220
 GTCGTCCAGGAGCTGCTGTGCACAATAGCGAGCCAGAGGCTACGGTGGCCGAGTAGCG
 L L D E V L R T I R E T E S A V R S M A
 A P G R R S T N D A R D G I G G P E D R
 C T R S S V H * G S P R R H W G A * R T^{26/43}
 V C D G L G I V P Y P L R Q F R V T T D
 C A T A S G S S P I R C V N S V * P R I
 V R R P R D R P L S A A S I P C N H G S
 GTGTGCGACGGCCTCGGGATCGTCCCCCTATCCGCTGCGTCAATTCCGTGTAACACGGAT
 2230 2240 2250 2260 2270 2280
 CACACGCTGCCGGAGCCCTAGCAGGGGATAGCGACGAGTAAAGGCACATTGGTGCCTA
 H A V A E P D D G I R Q T L E T Y G R I
 T R R G R S R G R D A A D I G H L W P D
 H S P R P I T G * G S R * N R T V V S R

FIGURE 6 (CONT'D)

R P E L P H L G G G V C V R F G H P D R
 D P S C P T S V E G F A S G S G T R T G
 T R V A P P R W R G L R Q V R A P G P V
 CGACCCGAGTTGCCCCACCTCGGTGGAGGGTTTGGCTCAGGTTCCGGCACCACCCGACCCGG
 2410 2420 2430 2440 2450 2460
 GCTGGCTCAACGGGTGGAGCCACCTCCCCAAACGACGTCACAGCCCGTGGCCTGGCC
 S G L Q G V E T S P N A D P E P V R V P
 V R T A G G R H L P K R * T R A G P G T
 G S N G W R P P P T Q T L N P C G S R Y

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* L D L A A I Q R * V D D F A R G L R D
 S L T S P R S N G R S T T S L A V C A T
 A * P R R D P T V G R R L R S R F A R P
 TAGCTTGACCTCGCCGCGATCCAACGGTAGGTCGACGACTTCGCTCGCGGTTGCGCGAC
 2470 2480 2490 2500 2510 2520
 ATCGAACTGGAGCGCGCTAGGTTGCCATCCAGCTGCTGAAGCGAGCGCCAAACGCGCTG
 L K V E G R D L P L D V V E S A T Q A V
 A Q G R R S G V T P R R S R E R N A R G
 S S R A A I W R Y T S S K A R P K R S R

FIGURE 6 (CONT'D)

R R N G A S A R L M M T I P ' A V V A A T
 A A T A P A P A * * * R F R R S S R R P
 P Q R R Q R P L D D D D S G R R G D Q
 CGCCGCAACGGCCAGCGCCGCTTGATGATGACGATTCCGGCGGTCGTCGGCGGACC
 2530 2540 2550 2560 2570 2580
 GCGGCGTTGCCGCGTCCGGGCGAATACTACTAGCTAAGCCGCCAGCAGCGCGCTGG
 A A V A G A G A Q H H R N R R D D R R G
 G C R R W R G S S S S E P P R R P S W
 R L P A L A R K I I V I G A T T A A V L

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N A I T V T I P K M I S I C N I V A S T
 T Q S P * R F R K * S A S A T S W R R R
 R N H R D D S E N D Q H L Q H R G V D V
 AACGCAATCACCGTGACGATTCCGAAATGATCAGCACTCTGCAACATCGTGCGTCGACG
 2590 2600 2610 2620 2630 2640
 TTGCGTTAGTGCACTGCTAAGGCTTTTACTAGTCGTAGACGTTGTAGCACCGCAGCTGC
 V C D G H R N R F H D A D A V D H R R R
 R L * R S S E S F S * C R C C R P T S T
 A I V T V I G F I I L M Q L M T A D V N
 L P I D R P V T M T S C P F R L G A A S
 C P S T G R * R * R R A R F G S E R P A
 A H R Q A G D D D V V P V S A R S G Q H
 TTGCCCATCGACAGGCCGGTGACGATGACGTCGTGCCCGTTTCGGCTCGGAGCGGCCAGC

FIGURE 7

1 GAA TTC CAA CCG TCG GTG CAG ATC CAG GTC TAT CAG GGG GAG CGT GAG 48
Glu Phe Gln Pro Ser Val Gln Ile Gln Val Tyr Gln Gly Glu Arg Glu

49 ATC GCC GCG CAC AAC AAG TTG CTC GGG TCC TTC GAG CTG ACC GGC ATC 96
Ile Ala Ala His Asn Lys Leu Leu Gly Ser Phe Glu Leu Thr Gly Ile

97 CCG CCG GCG CCG CGG GGG ATT CCG CAG ATC GAG GTC ACT TTC GAC ATC 144
Pro Pro Ala Pro Arg Gly Ile Pro Gln Ile Glu Val Thr Phe Asp Ile

145 GAC GCC AAC GGC ATT GTG CAC GTC ACC GCC AAG GAC AAG GGC ACC GGC 192
Asp Ala Asn Gly Ile Val His Val Thr Ala Lys Asp Lys Gly Thr Gly

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FIGURE 7 (CONT'D)

193 AAG GAG AAC ACG ATC CGA ATC CAG GAA GGC TCG GGC CTG TCC AAG GAA 240
Lys Glu Asn Thr Ile Arg Ile Gln Glu Gly Ser Gly Leu Ser Lys Glu

241 GAC ATT GAC CGC ATG ATC AAG GAC GCC GAA GCG CAC GCC GAG GAG GAT 288
Asp Ile Asp Arg MET Ile Lys Asp Ala Glu Ala His Ala Glu Glu Asp

289 CGC AAG CGT CGC GAG GAG GCC GAT GTT CGT AAT CAA GCC GAG ACA TTG 336
Arg Lys Arg Arg Glu Glu Ala Asp Val Arg Asn Gln Ala Glu Thr Leu

337 GTC TAC CAG ACG GAG AAG TTC GTC AAA GAA CAG CGT GAG GCC GAG GGT 384
Val Tyr Gln Thr Glu Lys Phe Val Lys Glu Gln Arg Glu Ala Glu Gly

FIGURE 7 (CONT'D)

385 GGT TCG AAG GTA CCT GAA GAC ACG CTG AAC AAG GTT GAT GCC GCG GTG 432
Gly Ser Lys Val Pro Glu Asp Thr Leu Asn Lys Val Asp Ala Ala Val

433 GCG GAA GCG GAA GGC GGC ACT TGG CGG ATC GGA TAT TTC GGC CAT CAA 480
Ala Glu Ala Glu Gly Gly Thr Trp Arg Ile Gly Tyr Phe Gly His Gln

481 GTC GGC GAT GGA GAA GCT GGG CCA GGA GTC GCA GGC TCT GGG GCA AGC 528
Val Gly Asp Gly Glu Ala Gly Pro Gly Val Ala Gly Ser Gly Ala Ser

529 GAT CTA CGA AGC AGC TCA GGC GGC TGC GTC ACA GGC CAC TGG CGC TGC CCA 576
Asp Leu Arg Ser Ser Gly Cys Val Thr Gly His Trp Arg Cys Pro

577 CCC CGG CGG CGA GCC GGG CGG TGC CCA CCC CGG CTC GGC 615
Pro Arg Arg Arg Ala Gly Arg Cys Pro Pro Arg Leu Gly

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FIGURE 8

5' TCGAACGAGGGCGGTGACCCGGTCCGGGGCTTCTTGCACTCGGCATAGGCCGAGTGCTAAG
 3' AGCTTGCTCCCCGCACTGGGCCACGCCCCCGAAGAACGTGAGCCGTATCCGCTCACCGATT
 10 20 30 40 50 60
 AATAACGTTGGCACTCGCGACCGGTGAGTGCTAGGTCGGGACGGTGAGGCCAGGCCCGTC
 TTATTGCAACCGTGAGCGCTGGCCACTACGATCCAGCCCTGCCACTCCGGTCCGGGCAG
 70 80 90 100 110 120
 GTCGCAGCGAGTGGCAGCGGAGGACAACTTGAGCCGTCCTCGCGGGCACTGCGCCCCGGCC
 CAGCGTCGCTACCGTCGCTCCTGTTGAACTCGGCAGGCAGGCCCTGTGACGCGGGCCGG
 130 140 150 160 170 180
 * R G C R H P V T P V S S P I R R
 AGCGTAAGTAGCGGGTTGCCGTACCCCGGTGACCCCGTTCATCCCGATCCGGAGGA
 TCGCATTTCATCGCCCCAACGCGCAGTGGGCCACTGGGGCAAGTAGGGCTAGGCCTCCT
 190 200 210 220 230 240
 N H F A M A K T I A Y D E A R R G L E
 ATCACTTCGCAATGGCCAAGACAATTGCGTACGACGAAGAGGCCCGTCGCGGCCTCGAGC
 TAGTGAAGCGTTACCGGTTCGTGTTAACGCATGCTGCTCTCCGGGCAGCGCCGGAGCTCG
 250 260 270 280 290 300
 R G L N A L A D A V K V T L G P K G R N
 GGGGCTTGAACGCCCTCGCCGATGCGGTAAAGGTGACATTTGGGCCCCCAAGGCCCGCAACG
 CCCCCAATTGCGGAGCGGCTACGCCATTTCACCTGTAAACCCGGGGTTCCCGCGTTGC
 310 320 330 340 350 360
 V V L E K K W G A P T I T N D G V S I A
 TCGTCCTGGAAAAGAAGTGGGTGCCCCCAGCATCACCACGATGGTGTGTCCATCGCCA
 AGCAGGACCTTTTCTTACCCCCACGGGGGTGCTAGTGGTTGCTACCACACAGGTAGCGGT
 370 380 390 400 410 420

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FIGURE 8 (CONT'D)

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K E I E L E D P Y E K I G A E L V K E V
AGGAGATCGAGCTGGAGGATCCGTACGAGAAGATCGGCGCCGAGCTGGTCAAGAGGTTAG
TCCTCTAGCTCGACCTCCTAGGCATGCTCTTCTAGCCGCGGCTCGACCCAGTTCTCCATC
430 440 450 460 470 480
A K K T D D V A G D G T T A T V L A Q
CCAAGAAGACCGATGACGTGCGCCGTGACGGCACCCAGCGGCCACCGTGTGGCCCAAG
GGTTCTTCTGGCTACTGCAGCGGCCACTGCCGTGGTGTGCTGCGCGGTGGCACGACCGGTCC
490 500 510 520 530 540
A L V R E G L R N V A A G A N P L G L K
CGTTGGTTCCGAGGGCCTGCGCAACGTGCGCGCGGCCCAACCCGCTCGGTCTCAAAAC
GCAACCAAGCGCTCCCGGACGCGTTGCAGCGCGCGCGCGGTGGCGAGCCAGAGTTTG
550 560 570 580 590 600
R G I E K A V E K V T E T L L K G A K E
GCGGCATCGAAAGGCGGTGGAGAAGGTACCGAGACCCCTGCTCAAGGGCGGCCAAGGAGG
CGCCGTAGCTTTTCCGGCACCTCTTCCAGTGGCTCTGGGACGAGTTCCCGCGGTTCCTCC
610 620 630 640 650 660
V E T K E Q I A A T A A I S A G D Q S I
TCGAGACCAAGGAGCAGATTGCGGCCACCGCAGCGATTTCGGCGGTGACCCAGTCCATCG
AGCTCTGGTTCCCTCGTCTAACGCGCGGTGGCTCGCTAAAGCCGCCCTGCTCAGGTAGC
670 680 690 700 710 720
G D L I A E A M D K V G N E G V I T V E
GTGACCTGATCGCCGAGGCGATGGACAAGGTGGGCAACGAGGGCGTCAATCACCCTCGAGG
CACTGGACTAGCGGCTCCGCTACCTGTTCCACCCGTTGCTCCCGCAGTAGTGGCAGCTCC
730 740 750 760 770 780

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FIGURE 8 (CONT'D)

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E S N T F G L Q L E L T E G M R F D K G
AGTCCAACACCTTTGGGCTGCAGCTCGAGCTCACCGAGGGTATGCGGTTCGACAAGGGCT
790 800 810 820 830 840
TCAGGTTGTGGAACCCGACGTCGAGCTCGAGTGGCTCCCATACGCCAAGCTGTTCCCGA

Y I S G Y F V T D P E R Q E A V L E D P
ACATCTCGGGGTACTTCGTGACCGACCCGGAGCGTCAGGAGCGGTCTCTGGAGGACCCCT
850 860 870 880 890 900
TGTAAGCCCCCATGAAGCACTGGCTGGGCCCTCGCAGTCTCCGCCAGGACCTCCTGGGGA

Y I L L V S S K V S T V K D L L P L L E
ACATCCTGCTGGTCAGCTCCAAGGTGTCCACTGTCAAGGATCTGCTCCCGCTGCTCGAGA
910 920 930 940 950 960
TGTAAGACGACCCAGTCGAGGTTCCACAGGTGACAGTTCCTAGACGACGCGCAGCAGCTCT

K V I G A G K P L L I I A E D V E G E A
AGGTCAICGGAGCCGGTAAGCCGCTGCTGATCATCGCCGAGGACGTCGAGGCGCAGGCGC
970 980 990 1000 1010 1020
TCCAGTAGCCTCGGCCATTTCGGCGACGACTAGTAGCGGCTCCTGCAGCTCCCGCTCCCGC

L S T L V V N K I R G T F K S V A V K A
TGTCCACCCCTGGTCGTCACAAGATCCGCGGCACCTTCAAGTCGGTGGCGGTCAAGGCTC
1030 1040 1050 1060 1070 1080
ACAGGTGGGACCCAGCAGTTGTTCTAGGCGCCGTGGAAGTTCAGCCACCCGCCAGTTCGAG

P G F G D R R K A M L Q D M A I L T G G
CCGGCTTCGGCGACCCGCGCAAGCGGATGCTGCAGGATATGGCCATTCTCACCCGGTGGTC
1090 1100 1110 1120 1130 1140
GGCCGAAGCCGCTGGCGGCGTTCCGCTACGACGTCCTATACCCGTAAGAGTGGCCACCCAG

Q V I S E E V G L T L E N A D L S L L G
AGGTGATCAGCGAAGAGGTGCGGCTGACGCTGGAGAAGCCGACCTGTGCTGCTAGGCA
1150 1160 1170 1180 1190 1200
TCCACTAGTCGCTTCTCCAGCCGGACTGCGACCTCTTTCGGCTGGACAGCGCAGATCCGT

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FIGURE 8 (CONT'D)

K A R K V V V T K D E T T I V E G A G D
 AGCCCGCAAGGTCGTGTCACCAAGGACGAGACCACCATCGTCGAGGGCGCGGTGACA
 TCCGGCGGTTCCAGCACCAAGTGGTTCTGCTCTGGTGGTAGCAGCTCCCGCGGCCACTGT
 1210 1220 1230 1240 1250 1260
 T D A I A G R V A Q I R Q E I E N S D S
 CCGACGCCATCGCCGGACGAGTGGCCAGATCCGCCAGGAGATCGAGAACAGCGACTCCG
 GGCTGGGTAGCGGCCCTGCTCACCGGGTCTAGCGGTCCTCTAGCTCTTGTGCTGAGGC
 1270 1280 1290 1300 1310 1320
 D Y D R E K L Q E R L A K L A G G V A V
 ACTACGACCGTGAGAAAGCTGCAGGAGCGGCTGGCCAAAGCTGGCCGGTGGTGTGCGGGTGA
 TGATGCTGGCACTCTTCGACGTCTCGCCGACCGGTTTCGACCGGCCACACAGCGCCACT
 1330 1340 1350 1360 1370 1380
 I K A G A A T E V E L K E R K H R I E D
 TCAAGGCCCGTCCCGCCACCGAGGTGCAACTCAAGGAGCGCAAGCACCGCATCGAGGATG
 AGTTCGGGCCACGGCGGTGGCTCCAGCTTGAGTTCTCGCGTTCGTGGCGTAGCTCCTAC
 1390 1400 1410 1420 1430 1440
 A V R N A K A A V E E G I V A G G G V T
 CGGTTCCGCAATGCCAAGGCCCGCGTTCGAGGAGGGCATCGTCGCCGGTGGGGGTGTGACGC
 GCCAAGCGTTACGGTTCCGGCGGCAGCTCCTCCGTAGCAGCGGCCACCCACACTGCG
 1450 1460 1470 1480 1490 1500
 L L Q A A P T L D E L K L E G D E A T G
 TGTTCGAAGCGGCCCGACCCCTGGACGAGCTGAAGCTCGAAGGCGACGAGCGACCGCGG
 ACAACGTTCCCGCGGGCTGGGACCTGCTCGACTTCGAGCTTCGCGTCTCCGCTGGCCGC
 1510 1520 1530 1540 1550 1560
 A N I V K V A L E A P L K Q I A F N S G
 CCAACATCGTGAAGGTGGCGCTGGAGGCCCGCTGAAGCAGATCGCCTTCAACTCCGGGC
 GGTGTAGCACTTCCACCGCGACCTCCGGGGGACTTCGTCTAGCGGAAGTTGAGGCCCG
 1570 1580 1590 1600 1610 1620

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FIGURE 8 (CONT'D)

L E P G V V A E K V R N L P A G H G L N
TGGAGCCGGCGTGGTGGCCGAGAGGTGCGCAACCTGCCGGCTGGCCACGGACTGAACG
ACCTCGGCCCCGACACCGGCTCTTCCACGCGTTGGACGCGCCGACCGGTGCTGACTTGC
1830 1840 1850 1660 1670 1680

A Q T G V Y E D L L A A G V A D P V K V
CTCAGACCGGTGTCTACGAGGATCTGCTCGCTGCCGGCGTTGCTGACCCGGTCAAGGTGA
GAGTCTGCCACACAGATGCTCCTAGACGAGCGACGCGCCGCAACGACTGGGCCAGTTCCACT
1690 1700 1710 1720 1730 1740

T R S A L Q N A A S I A G L F L T T E A
CCCCGTTCCGGCTGCAGAAATGCGGCGTCCATCGCGGGGCTGTTCTTGACCCACCGAGGCCG
GGCAAGCCCGGACGTCTTACGCGCGAGGTAGCGCCCCGACAAAGGACTGGTGGCTCCGGC
1750 1760 1770 1780 1790 1800

V V A D K P E K E K A S V P G G D M G
TCGTTCCCGACAAAGCCGGAAGGAGAGGCTTCCGTTCCCGGTGGCGGCGACATGGGTG
AGCAACGGCTGTTCGGCCTTTTCTCTTCCGAAGGCAAGGCCACCGCGCTGTACCCAC
1810 1820 1830 1840 1850 1880

G M D F *
GCATGGATTCTGACCCCGCGGAGAGTCCGACGCGAGGAGCCCGTCCCTTTGTGGGGCC
CGTACCTAAAGACTGGGGCCGCTCTTCAGCGTCGCTCCTCGGGCCAGGGAACACCCCGG
1870 1880 1890 1900 1910 1920

GGGCTCCTCTGGTGGAGCTACGGTACCGAGAACACCAACGAGTCGTGTAGGCAACCTT
CCCGAGGAGACCAACCTCGATGCCATGGCTCTTGTGTGCTCAGCACATCCGTTGGAA
1930 1940 1950 1960 1970 1980

TGGCCGCTGTGGCGAGTCGGGGCCCGCTCTCGGTGCAGCAGCGCGGATGGGTACGA
ACCGGCGACACCGCTCAGCCCCCGGCGCAGAGCCACGTCGTGCGCGGCTACCCATGCT
1990 2000 2010 2020 2030 2040

FIGURE 8 (CONT'D)

CACCGCAGCGGGGTGTCGTCATCGGGGCCCTGCGTCCGACGCCCTGGGCACGGCCGTCGA
 GTGGCGTCGCCCGCCACAGCAGTAGCCCCCGGACGCGAGGCTGCGGACCCGTGCCGCGCAGCT
 2050 2060 2070 2080 2090 2100
 CGATCAGCGAGTAGCCCGCTAGGATCGGATGGCGGCCACAACAGGGTGACTTCGCTGCGGT
 GCTAGTCGCTCATCGGCGATCCTAGCCTACCGCCGGTGTGTCTCCACTGAAGCGACGCCA
 2110 2120 2130 2140 2150 2160
 GGGCCAGGTTTTGCCCGGTACGACCCCCGATCAGGCCGACGTCGACCACTGCCCGGGGTC
 CCCGGTCCAAACGGCGCATGCTGGGGCTAGTCCGGCTGCAGCTGGTGACGGGCCCCAG
 2170 2180 2190 2200 2210 2220
 CATCGGGGCCGTCGGGGAGTTCGGCGCAGCACCGGCTCGACTGCCACCGTGTGCACGCGAT
 GTAGCCCCGGCAGCCCCCTCAAGCGCGTCTGGCCGAGCTGACGGTGCCACACGTGCGCTA
 2230 2240 2250 2260 2270 2280
 2290 2300 2310 2320 2330 2340
 GGCCATCATCGACGGTGATCAGGTAAAGCGAACGGGTAGTCGGGCAAGCGCGGCCAGCC
 CCGGTAGTACCTGCCACTAGTCCATTGCGTTGCCCATCAGCCCCGTTCCGCCCGCGGTCGG
 2350 2360 2370 2380 2390 2400
 GTTTGAGGCTACCTTTTGGCACCCACCGATTTCGAGGATAGGCGCCCGATGTGTTACTC
 CAACTCCAGATGGAAACCGTGGTGCCCTAAGCTCCTATCCGCGGGCTACACAATGAG
 2410 2420 2430 2440 2450 2480
 CGAACCGACCGGCTGCCCGATCCGCGGGCTGGCGTAGGCGGATTTCGGCGTCCGGGCTCGG
 GCTTGGCTGGCCGACGGGCTAGGCGGCCCGACCGCATCCGCCCTAAGCGCCAGCCCCGAGCC
 S G V P Q G I R P S A Y A S E R D P S P
 2470 2480 2490 2500 2510 2520
 GTAGAAGTTCGACTTGGGGATGCCGAGCCGGGGGTACTCGGCTCAGCGCAGCGCGGTATT
 CATCTTCAAGCTGAACCCCTACGGCCTCGGCCCCCATGAGCCGAGTGGCTGCCGCCATAA
 Y F N S K P I G S G P I S P E R V A T H

FIGURE 8 (CONT'D)

2530 2540 2550 2580 2570 2580
 CCGCAAGCCCGAGTCGTTGCTGCCCGGAGTTGACGAAGCTCGGGTAGCTGGTGCCAGGGCT
 GCGGTTCCGGGCTCAGCAACGACGGGCTCAACTGCTTCGAGGCCATCGACCACGGTCCCCGA
 R L G S D N S G S H V F S P Y S T G P S

 2590 2600 2610 2620 2630 2640
 TCTAAGCCCGGGGTTTGCCGCCGAGCCAGCCGCGGCACTGCCGCTACCGGGTTCCGGGTT
 AGATTCCGGGCCCAACGCGGCTCGGTGCGCGCGCTGACGGCGATGGCCCAAGCCCAA
 R L G P N A G S G A A S G S G P N P N

 2650 2660 2670 2680 2690 2700
 GCCTGAGTCCAGGCCGCCAACAGGAGCACTGGCCGGGCGGCGACGGCGTGTGGTTCAG
 CGGACTCAGGTCCGGCGGTTGTCTCTGTCGACCGGCCCGCGCTGCCCGCACACACAGTC
 G S D L G G V P A S A P A A V P T N T L

 2710 2720 2730 2740 2750 2760
 GCGGAGTTGAGGACGTTCCGCCAGGCCGTTGTGGAGACCGCCCGTTGATCCGAGGGCGGA
 CCGGCTCAACTCCTGCAAGCGGTCCGGCACAACTCTGCGGGCACTAGGCTCCCCGCT
 G S N L V N A L G H Q L G G T S G L A S

 2770 2780 2790 2800 2810 2820
 GCGGAGGATGCCCGCAACTCAAAGCCGCCGTGCTCATGCCGCCGGTGGCGTAGCCGGCGGA
 CCGCTCCTACGGGCTTGAGTTTCGGCGGCACGAGTACGGCGGCCACCGCATCGGCCGCT
 A L I G S S L A A T S M G G T A Y G A S

 2830 2840 2850 2860 2870 2880
 GGTGACCAAGGCCGCTCCGAGCCAGCCCGCGCTTCCTAAGGCGGCGTTTTCATCCCCGC
 CGACTGGTTCCGGCGGAGGCTCGGTGCGCGCGAAGGATTCCGCCGCAAAACGTAGGGCGG
 S V L A A E S G A A S G L A A N Q M G A

 2890 2900 2910 2920 2930 2940
 GTTCCAGAAGCTGGTGTGAGGCTGCCCTGCGCTGCCGAGGCCCGCGTTGATTGTCCCCGA
 CAAGGTCTTCGACCCACAACCTCCGACGCGACGCGCTCCGGCGCAACTAACAGGGGCT
 H W F S T H L S G A S G L G A N I T G S

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FIGURE 8 (CONT'D)

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2950      2980      2970      2980      2990      3000
GGTCCCGATGCCCGCTGTTACGGGAGCCCGAATTCCCGATGCCGATGTTCCCGCTGCCCGGA
CCAGGGCTACGGCGACAAAGTCCCTCGGGCTTAAGGGCTACGGCTACAAAGCGCAGCGCCT
T G I G S H L S G S H G I G I H G S G S

3010      3020      3030      3040      3050      3060
GTTGAATAAGCCGACGTTGCCGGTGCCCGAGTTCCCGAAGCCGATGTTGCCGCTACCCGA
CAACTTATTCGGCTGCAACGGCCACGGGCTCAAGGCTTCGGCTACAAACGGCGATGGGCT
N F L G V N G T G S N G F G I N G S G S

3070      3080      3090      3100      3110      3120
GTTGAAGCCGCCGAAACCCATCTGTGATCACCGGTGATCCCGAACC CGATATTCCCGCT
CAACTTCGGCGGCTTTGGGTAGACCACCTAGTGGCCACTAGGCTTGGGCTATAAGGGCGA
N F G G F G M Q H D G T I G F G I N G S

3130      3140      3150      3160      3170      3180
ACCGGTGTTGCCGAAGCCGATATTCCCGTCGCCGAGGTTGCCGAGGCCAGGTTGCCGCT
TGGCCACAACGGCTTCGGCTATAAGGGCAGCGGCTCCAAACGGCTCCGGGTCCAACGGCGA
G T N G F G I N G D G L N G L G L N G S

3190      3200      3210      3220      3230      3240
GCCGGTGTGCCCGCTGCCGATGTTGCCGGTGCCGGTGTTGCCGCTGCCGATGTTGTTGTT
CGGCCACAACGGCGACGGCTACAACGGCCACGGCCACAACGGCGACGGCTACAACAACAA
G T N G S G I N G T G T N G S G I N N N

3250      3260      3270      3280      3290      3300
GCCGATGTTGTTGCCGATGTTGTTGTTGCCGATGTTGCCGCTGCCGGTGTGTTGCCGAA
CGGCTACAACAACGGCTACAACAACGGCTACAACGGCGACGGCCACCAACGGCTT
G I N N N G I N N N G I N G S G T N G F

3310      3320      3330      3340      3350      3360
GCCCAGATIGATCTGGCCGTTCTTGCCGATGTCGATGCCGAGGTTCCCGCAAGACCTGCTG
CGGGTCTAAGTACCGGCAAGAACGGCTACAGCTACGGCTCCCAAGGCGTCTGACGAC
G L N I Q G N K G I D I G L N R L V Q .Q

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FIGURE 8 (CONT'D)

3370 3380 3390 3400 3410 3420
 CCAGGGCGCCAGTTGTGCGACGGCCGACGCGCATCGAAGTGGTAACAGCCATCGCCGC
 GGTCCCGCGGTCAACACGCTGCCGCGTCTGCGTAGCTTACCATTTGGTCCGTAGCGGCG
 W P A L Q A V A A S A D F H Y G A M A A
 3430 3440 3450 3460 3470 3480
 CACGTCCAATGCCACATTTGCTCGTATGCCGCCCTCGACGTCCATGAGCGCCGAGCGTT
 GTGCAGGTACGGGTGTAAACGAGCATACGGCGAGCTGCAGTACTCGCGGCTCGCAA
 V D L A W M Q E Y A A E V D M L A P A N
 3490 3500 3510 3520 3530 3540
 CTGCCCAAACAGTTTCGTAGCTGCCAGCAGCTGCATCAGGCCACGATTGGCCGCTACCCAC
 GACGGGTTTGGTCAAGCATCGACGCTCGTCGACGTAGTCCGGTGCTAACCCGGCGATGGTG
 Q G F W N T A A L L Q M L G R N A A V V
 3550 3560 3570 3580 3590 3600
 TGCCGGCTGCACGGTGGCCCGCCAGCGCCCGCTCGAACGCGGTGCTGTGCCATGGCCCTG
 ACGCCGACGTGCCACCGCGGTCGCGGCGGAGCTTGCGCCAGCGACAAACGGTACCGGAC
 A P Q V T A A L A A E F A T A T A M A Q
 3810 3820 3830 3840 3850 3860
 TGCCGGCGCTTGTTCGGCTGCGCTGCCCGCGTCTGAGCCAGGCTAGGTACTGGGTTC
 ACGCCGGGAACAAGCGGACGCGACGCGGCGGACGACTCGGTCCGATCCATGACCCAAACG
 A A A Q E A Q A A A T S L W A L Y Q T A
 3870 3880 3890 3900 3910 3920
 GACGGCCATCATCGCCCGCGGACGCGACCCAGCCAGCGCGCCACTAGTCAGTTCGGAIGT
 CTGCCGGTAGTAGCGCGCGGCTGCTGCGGTCCGCGGTGATCAGTCAAGCCTACA
 V A M M A A A S P G L W A G S T L E S T
 3730 3740 3750 3760 3770 3780
 GACGGAGCCAAAGCGACGCTATTGACCGGAGCAATTCTTCGGCCAGCTCGCCCCAGGCGGT
 CTGCCCTCGGTTCGCTCGGATAACTGCGCTCGTTAAGAACCGGTCGAGCGGGTCCGCCA
 V S G L S A I S A L L E E A L E G W A T

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FIGURE 8 (CONT'D)

3790 3800 3810 3820 3830 3840
 GGCCGACGCAATTAGCGGTCCCGACCCGGGACCGCAACATCAGTGCCGAAATTGATCTC
 CCGGCGTCGTTAATCGCCAGGCTGGGCCCTGGCCGTTTGTAGTCACGGCTTAACCTAGAG
 A A A I L P G S G P G A F M L A S N I E
 3850 3860 3870 3880 3890 3900
 TGGCGGCAACCAACGCAAAATGCGGGCTTGTACGCCGATCCAACTTAACGTGTCAGCGACCG
 ACCGCCGTTGGTGGCTTTTACGCCCGCAACAGTCGGCTAGGTTGAATTGACAGTCGCTGGC
 P P L W A F H P S T L R D L K V T L S R
 3910 3920 3930 3940 3950 3960
 TTGCCGTGGCGGTATCGGCACCTTCAATACCACTCATCTTTGGGGTCACTTTGGAGCGCC
 AACGGCACCGCCATAGCCGTGAAGTTATGGTGAGTAGAAACCCAGTAGAAACCTCGCGG
 Q R P P I P V E I G S M K P T M K P A G
 3970 3980 3990 4000 4010 4020
 CCTAGGAACCGCCAGCTTACCTAGTCCCGGGTAGGGCCGACTGGCGGCCGGGATGTCAGC
 GGATCCTTGGCGGTCGAATGGATCAGGGCCCATCCCGGCTGACCGCCGCCCTACGTGC
 R P V A L K G L G P Y P G V P P R S A A
 4030 4040 4050 4060 4070 4080
 TGAGGGTCTGCCACCTGCC'CGTAATGTCGCTGGTATGGCAAGCACCGACCGCCGGCGCC
 ACTCCAGACGGTGGACGGGGCATTTACAGCGACCATACCGTTCTGCTGGCTGCGGCGCCGG
 S P R G G A G Y H R Q Y P L C R R R P G
 4090 4100 4110 4120 4130 4140
 AAGAGTTCCTCCGCGACGCGTTACCCCGGTTGATCGAACATGTGACGAACTCACCACCG
 TTCTCAACGAGCGCTGCGCAAGTGGGCCAACTAGCTTGTACAGCTGCTTGAGTGGCTGC
 L T A G R R T *
 4150 4160 4170 4180 4190 4200
 GCCTCACCGACCAACTCGCCTGCTACCGCCCGACCCCGGCGCCCAACAGCATTTGCGTGGC
 CGAGTGGCTGGTTGAGCGGACGATGGCGGGCTGGGGGTGCGGTTGTCGTAACGACCGC

FIGURE 8 (CONT'D)

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4210 4220 4230 4240 4250 4280
TGCTCTGGCACAGCGCCCGGTGCAGGATATACAGGTCGCCCATGTGGCCGGCGTGGAAG
ACGAGACCGTGTGCGGGCCACGTCCTATATGTCCAGCGGTACACCGCGCCGACCTTC

4270 4280 4290 4300 4310 4320
AGGTGTGGACCCCGGACGGTTGGGTGGACCGCTTTGGGTTAGATCTGCCCGGCGCACGACA
TCCACACCTGGGCGCTGCCAACCACCTGGCGAAACCCAACTCTAGACGGCGCGCTGTGT

4330 4340 4350 4360 4370
CCGGATATGGACACCGTCCCGAGGATGTGGCGAAGGTACGGGCACCCCGCGGGAATTC 3'
GGCCTATACCTGTGGCAGGGCTCCTACACCGCTTCCATGCCCGTGGCGGCTGCCCTTAAG 5'
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification⁴ : C12N 15/00, A61K 39/04 G01N 33/569</p>	<p>A3</p>	<p>(11) International Publication Number: WO 88/ 05823 (43) International Publication Date: 11 August 1988 (11.08.88)</p>
<p>(21) International Application Number: PCT/US88/00281 (22) International Filing Date: 1 February 1988 (01.02.88) (31) Priority Application Number: 010,007 (32) Priority Date: 2 February 1987 (02.02.87) (33) Priority Country: US (71) Applicant: WHITEHEAD INSTITUTE FOR BIOM- EDICAL RESEARCH [US/US]: Nine Cambridge Center, Cambridge, MA 02142 (US). (72) Inventors: HUSSON, Robert, N. : 60 Parkman Street, Brookline, MA 02146 (US). YOUNG, Richard, A. : 11 Sussex Road, Winchester, MA 01890 (US). SHIN- NICK, Thomas, M. : 1434 Rainier Falls Drive, Atlan- ta, GA 30329 (US).</p>	<p>(74) Agents: GRANAHAH, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lex- ington, MA 02173 (US). (81) Designated States: AT (European patent), AU, BE (Eu- ropean patent), CH (European patent), DE (Euro- pean patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European pa- tent), NL (European patent), SE (European patent). Published <i>With international search report</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i> (88) Date of publication of the international search report: 3 November 1988 (03.11.88)</p>	
<p>(54) Title: MYCOBACTERIUM TUBERCULOSIS GENES ENCODING PROTEIN ANTIGENS</p> <p>(57) Abstract</p> <p><i>Mycobacterium tuberculosis</i> genes encoding five immunologically relevant proteins have been isolated by systemati- cally screening a lambda gt11 recombinant DNA expression library with a collection of murine monoclonal antibodies di- rected against protein antigens of this pathogen. One of the <i>M. tuberculosis</i> antigens, a 65kD protein, has been shown to have determinants common to <i>M. tuberculosis</i> and <i>M. leprae</i>. In addition, genes encoding proteins of other mycobacteria (<i>M. africanum</i>, <i>M. smegmatis</i>, <i>M. bovis</i> BCG and <i>M. avium</i>) have been isolated. Isolation and characterization of genes en- coding major protein antigens of <i>M. tuberculosis</i> make it possible to develop reagents useful in the diagnosis, prevention and treatment of tuberculosis. They can be used, for example, in the development of skin tests, serodiagnostic tests and vaccines specific for tuberculosis.</p>		

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FI	Finland				

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 88/00281

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : C 12 N 15/00; A 61 K 39/04; G 01 N 33/569																	
II. FIELDS SEARCHED <div style="text-align: center;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 20%; border: none;">Classification System </td> <td style="border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none;">IPC⁴</td> <td style="border: none;">C 12 N; A 61 K; G 01 N</td> </tr> </table> <div style="text-align: center; padding-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	IPC ⁴	C 12 N; A 61 K; G 01 N											
Classification System	Classification Symbols																
IPC ⁴	C 12 N; A 61 K; G 01 N																
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border: none;"> <tr> <th style="width: 10%; border: none;">Category ⁹</th> <th style="width: 60%; border: none;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 30%; border: none;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="border: none; vertical-align: top;">X</td> <td style="border: none; vertical-align: top;">Proc. Natl. Acad. Sci. USA, volume 82, May 1985, R.A. Young et al.: "Dissection of Mycobacterium tuberculosis antigens using recombinant DNA", pages 2583-2587 see the whole document cited in the application</td> <td style="border: none; vertical-align: top;">1,2,4,6, 7,12-15, 19,23,25-27</td> </tr> <tr> <td style="border: none; vertical-align: top;">Y</td> <td style="border: none; vertical-align: top;">--</td> <td style="border: none; vertical-align: top;">3,8-11,20-22,24</td> </tr> <tr> <td style="border: none; vertical-align: top;">X</td> <td style="border: none; vertical-align: top;">Infection and Immunity, volume 51, no. 2, February 1986, American Society for Microbiology, (Washington, DC, US), H.D. Engers et al.: "Results of a World Health Organization-sponsored workshop to characterize antigens recognized by Mycobacterium-specific monoclonal antibodies", pages 718-720 see page 718, column 2, line 8 - page 719, column 1; tables I,II cited in the application</td> <td style="border: none; vertical-align: top;">1-16,19, 23-27</td> </tr> <tr> <td style="border: none; vertical-align: top;">Y</td> <td style="border: none; vertical-align: top;">--</td> <td style="border: none; vertical-align: top;">20-22</td> </tr> </table>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	Proc. Natl. Acad. Sci. USA, volume 82, May 1985, R.A. Young et al.: "Dissection of Mycobacterium tuberculosis antigens using recombinant DNA", pages 2583-2587 see the whole document cited in the application	1,2,4,6, 7,12-15, 19,23,25-27	Y	--	3,8-11,20-22,24	X	Infection and Immunity, volume 51, no. 2, February 1986, American Society for Microbiology, (Washington, DC, US), H.D. Engers et al.: "Results of a World Health Organization-sponsored workshop to characterize antigens recognized by Mycobacterium-specific monoclonal antibodies", pages 718-720 see page 718, column 2, line 8 - page 719, column 1; tables I,II cited in the application	1-16,19, 23-27	Y	--	20-22
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Y	--	3,8-11,20-22,24															
X	Infection and Immunity, volume 51, no. 2, February 1986, American Society for Microbiology, (Washington, DC, US), H.D. Engers et al.: "Results of a World Health Organization-sponsored workshop to characterize antigens recognized by Mycobacterium-specific monoclonal antibodies", pages 718-720 see page 718, column 2, line 8 - page 719, column 1; tables I,II cited in the application	1-16,19, 23-27															
Y	--	20-22															
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 50%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> Date of the Actual Completion of the International Search 3rd June 1988 </td> <td style="width: 50%; border: none; vertical-align: top;"> Date of Mailing of this International Search Report <div style="text-align: center; font-size: 1.2em;">07 OCT 1988</div> </td> </tr> <tr> <td style="border: none; vertical-align: top;"> International Searching Authority EUROPEAN PATENT OFFICE </td> <td style="border: none; vertical-align: top;"> Signature of Authorized Officer <div style="text-align: center;"> P.C.G. VAN DER PUTTEN </div> </td> </tr> </table>			Date of the Actual Completion of the International Search 3rd June 1988	Date of Mailing of this International Search Report <div style="text-align: center; font-size: 1.2em;">07 OCT 1988</div>	International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer <div style="text-align: center;"> P.C.G. VAN DER PUTTEN </div>											
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International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer <div style="text-align: center;"> P.C.G. VAN DER PUTTEN </div>																

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Chemical Abstracts, volume 105, no. 19, 10 November 1986, (Columbus, Ohio, US), S. Bhattacharya et al.: "Expression of Mycobacterium tuberculosis genes in Escherichia coli", see page 198, abstract 166229t, & J. Biosci. 1986, 10(2), 277-81 --	1-3,6-16, 23-27
X	Biological Abstracts/RRM, no. 30116549, T.M. Buchanan et al.: "Recombinant Escherichia-coli for expression and production of species-specific epitopes of Mycobacterium-leprae and Mycobacterium-tuberculosis", see title and terms, & Abstracts of the Annual Meeting of the American Society for Microbiology (US), 1986, vol. 86, no. 0, p. 122 --	14
X	Biological Abstracts, volume 82, no. 2, 1986, (Philadelphia, PA, US), B.R. Bloom et al.: "Genes for the protein antigens of the tuberculosis and leprosy bacilli", see page AB-532, abstract 14488, & Biosci. Rep. 5 (10/11): 839-846, 1985 --	1-27
Y	Nature, volume 316, 1 August 1985, (London, GB), R.A. Young et al.: "Genes for the major protein antigens of the leprosy parasite Mycobacterium leprae", pages 450-452 see the whole document cited in the application --	3,8-11,16, 19-22,24
Y	Nature, volume 319, no. 6048, 2 January 1986, (London, GB), A.S. Mustafa et al.: "Human T-cell clones recognize a major M. leprae protein antigen expressed in E. coli", pages 63-66 see the whole document cited in the application --	15
A	Infection and Immunity, volume 49, no. 2, August 1985, American Society for Microbiology, T.P. Gillis et al.: "Immunochemical characterization of a protein associated with Mycobacterium leprae cell wall", pages 371-377 -- ./...	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
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A	Infection and Immunity, volume 50, no. 3, December 1985, American Society for Microbiology, J.E.R. Thole et al.: "Cloning of Mycobacterium bovis BCG DNA and expression of antigens in Escherichia coli", pages 800-806 cited in the application	
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P,X	Infection and Immunity, volume 55, no. 6, June 1987, American Society for Microbiology, D.B. Young et al.: "Screening of a recombinant Mycobacterial DNA library with polyclonal antiserum and molecular weight analysis of expressed antigens", pages 1421-1425 see the whole document	1-27
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P,X	Proc. Natl. Acad. Sci. USA, volume 84, March 1987, R.N. Husson et al.: "Genes for the major protein antigens of Mycobacterium tuberculosis: The etiologic agents of tuberculosis and leprosy share an immunodominant antigen", pages 1679-1683	1-27
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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

Please see form PCT/ISA 206 sent to you on July 5th, 1988.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
1-16, 19-27
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

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